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Preliminary Characterization Of The Microbial Community In The Bonneville Salt Flats

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PRELIMINARY CHARACTERIZATION OF THE MICROBIAL COMMUNITY IN THE BONNEVILLE SALT FLATS

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ABSTRACT

Hypersaline environments (salt concentrations greater than two molar) are found on all continents and represent a woefully understudied ecosystem. To date, most of the hypersaline environments studied have been either lakes (e.g. The Great Salt Lake, Utah) or deep ocean/sea brine pools (e.g. Red Sea), both of which have an abundance of accessible water for the organisms living there. However, more extreme hypersaline environments have not been characterized as thoroughly. One of these environments is the Bonneville Salt Flats (BSF) in Western Utah. The BSF is 80 square miles in size and consists of a salt crust of varying thickness (0.5 inches to 5 feet) situated on top of a shallow brine aquifer. It experiences intense radiation and its only sources of water are meteorological events and upwelling from a shallow brine aquifer. The combination of high salt, water scarcity, and intense radiation provides an interesting mix of selection pressures and were the common reasons given as to why the BSF was thought to have no extant life. My work, presented here, was the first to report that there is extant life in the BSF and helps provide a better understanding of the structure and dynamics of the microbial population contained within this extreme type of saline environment. I began by growing isolates on various types of media containing four molar salt to replicate the environment and maximize the possibility of isolating true microbial inhabitants of the

environment. During this process I observed a variety of colony morphologies, cell shapes, pigments, and other external features. I then selected a subset of the organisms and began determining the temperature growth range/kinetics for each organism. The genus/species of each organism was also determined through PCR amplification and sequencing of the 16S rRNA gene. Finally, I performed analyses to link the phylogenetics of each organism to its native niche in the BSF in an attempt to determine how the microenvironments select for different genera. My analysis of the isolates will help elucidate and further characterize the microorganisms thriving in this polyextremophilic ecosystem and the effects of the selection pressures present in this environment.

Keywords: Phylogenetics, Polyextremophilic, Hypersaline, Microenvironments

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DEDICATION

I would like to dedicate this work to my dad, Dr. James Boogaerts, who cultivated my passion for science. A love of knowledge is perhaps the greatest gift one can bestow on another, and for this I thank you, dad. I would also like to dedicate this work to the love of my life, Ariana Diamond. Her warmth and eternal optimism were an ever-giving source of encouragement and support.

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INTRODUCTION

Prior to 1977, Scientists divided all life amongst five Kingdoms (Animalia, Fungi, Monera, Plantae, Protista). Under this classification system, the prokaryotic cells: bacteria and archaea, were classified under one subkingdom. However, this organization changed due to the pioneering genetic work of Carl Woese, who painstakingly decoded the DNA sequence of the RNA molecule (16S/18S rRNA) found within the small ribosomal subunit. This work led Woese to hypothesize that there were not five Kingdoms but three Domains of Life (Bacteria, Archaea, and Eukarya). Members of what would later become known as the Domain Archaea were not simply Bacteria that lived in extreme conditions (as previously thought), but a completely separate Domain of Life (1). Previous work in lipid/membrane biochemistry also supported Woese's hypothesis in that the Archaeal membrane contains L-glycerol (compared to D-glycerol found in the other two Domains) (2) that are ether bonded to lipids comprised of isoprene units (compared to ester bonded to phospholipids) (2, 3).

To date, Archaeal cells have been observed in a multitude of environments such as the ocean, soil, and deep subsurface. However, the most well characterized species were originally isolated from extreme environments (acidic, alkaline, high/low temperature, saline, etc.) where they often dominate. In fact, the results of the evolutionary pressures of these extreme environments are still at the heart of the main interest in these organisms. Two of the main sources of interest are (1) the

biotechnological applications of the biomolecules that result from living in an extreme environment and (2) their ability to be used as model organisms for extraterrestrial life.

Homeostasis

The ability of many archaea to live in extreme conditions has allowed them to thrive in conditions other organisms cannot. This niche at the extremes has caused these members of the Archaea to evolve in distinct ways and flourish under 'harsh' conditions; however, this also comes at the cost of not being able to survive less extreme environmental conditions (2, 4). The inability to live in less extreme environments shows how deeply rooted the adaptations needed to achieve homeostasis in extremes are. The pressure on cells to maintain homoeostasis in these environments is thought to be the main pressure that acted on Archaea into separation from the other domains, (2, 4, 5). This separation can be seen in their unique genetics, cellular membranes, and further individual adaptations. It is this ability, to keep homeostasis at a very low energy cost in any environment, that has defined Archaea as a domain.

Defining Characteristics

The members of the Domain Archaea differ in many ways but they also share defining characteristics that helped in outlining them as a Domain. These homologous characteristics are shared due to similar selection pressures faced by Archaea as a whole. While they live in different environments, it seems that there are some specific evolutionary adaptations that helped them adapt to specific extremes and thrive in those conditions (2, 4, 5).

Archaeal Membrane

The Archaeal membrane is perhaps the most recognized adaptation to extreme environments that all Archaea share and is different than what is found in either the Bacteria or Eukarya Domain. Interestingly, this knowledge actually predates Woese's pioneering work by about ten years (6). All living cells are surrounded by a membrane whose main purpose is to create a barrier defining what is inside and outside of the cell. The other role of the membrane is selective permeability, i.e. determining what is allowed to enter and exit the cell. Membrane permeability is a cell's main method of controlling its internal environment (homeostasis) in relation to its outside environment. The greater the difference between the external and internal environment of the cell, the harder it becomes to keep cellular homeostasis. It is the selective pressure of keeping cellular homeostasis in extreme environments that is thought to be the main driving force behind the difference found in the Archaeal membrane

Cells found in the Domains Bacteria and Eukarya are surrounded by a phospholipid membrane, as are any organelles within the cell. This membrane is made up of two layers, each of which is comprised of a head, consisting of glycerol and phosphate (hydrophilic), and a tail, made up of fatty acid chains (hydrophobic). These units, when placed in an aqueous solution, create a bilayer membrane with the hydrophobic groups facing in and the hydrophobic groups facing out.

The fatty acids are attached to the glycerol, found in the head, by an ester bond (2, 7). The phospholipid molecules are attracted to each other but are not connected which allows for fluidity of the membrane. This fluidity is described as the membrane existing

in a liquid crystalline phase (8). This phase describes the motion of both the membrane and the flow of material across it (2, 8). All three Domains contain proteins that help control this but the membrane itself is also permeable. The specific components of the membrane, such as differing fatty acids, can change this permeability.

Archaeal membranes are also made up of a phospholipid membrane, but the basic building blocks are much different. It is comprised of a glycerol-phosphate head (hydrophilic) and isoprene tails (hydrophobic) (2). These units align similarly to the other Domains with the hydrophobic units facing in and the hydrophilic units facing out. The isoprene tails attach to the glycerol backbone by an ether bond as opposed to an ester bond (2, 7). The glycerol found in the head of the archaeal membrane is glycerol-1-phosphate, which differs from the other Domains, which contain glycerol-3-phosphate (8). These are enantiomers and are made by the non-homologous enzymes, G1P dehydrogenase and G3P dehydrogenase (2, 7-9). Archaeal membranes are both bilayer and monolayer depending on the lipid core. The two most common lipids found in the Archaeal membrane are archaeol and caldarchaeol (8). Archaeol based membranes make bilayers and caldarchaeol based membranes form monolayers that feature 5-6 carbon ring structures (7).

Each of these differences directly affects cellular homeostasis and membrane integrity. Ether bonds are more stable than ester bonds and have been shown to keep better membrane integrity at higher temperature (10). Phenol based membranes stay in a liquid crystalline phase over a greater temperature range than fatty acid based ones (9, 10). As a result Archaea are able to keep a stable membrane with a constant rate of permeability without having to change the interior of their membrane (8, 10). This

strengthened membrane allows Archaea to achieve and conserve homeostasis with no more energy than it takes to make the initial membrane. Fatty acid based membranes have a much shorter range in which they can hold a liquid crystalline phase and must constantly change their membrane to adjust to an extreme temperature (8, 10). This shared adaptation allows Archaea as a whole to keep both stable membrane integrity and constant low permeability. This is a defining feature of Archaea and is one of the ways they have adapted to living in extreme environments.

Archaeal Genetic Structure and Information Transfer

The genetic architecture of Archaea is often described as simple Eukaryotic machinery working on Bacterial scaffolding (2). This is because the actual DNA of Archaea is very similar to Bacterial DNA and the machinery used to replicate and fix Archaeal DNA is very similar to what is found in Eukaryotes. Both Archaea and Bacteria contain circular DNA as a main chromosome and plasmids with each having important roles to the survival of the organism (11). This is different from Eukaryotes which have linear DNA that is tightly wound around histone proteins forming a dense structure called a nucleosome (2). These proteins regulate replication by changing the histones affinity for DNA through acetylation and phosphorylation of the histone (12). Archaea also have been shown to contain histone-like proteins, however, Eukaryotic like control of DNA replication regulation has not been clearly demonstrated in Archaea (13, 14). It has been shown that their histone-like proteins can be both acetylated and phosphorylated, but how or if they use this for replication regulation is still not known (12).

Bacteria and Archaea share A-T rich origins of replication that contain conserved elements (Bacteria: DnaA boxes and Archaea: ORC/Cdc6-like proteins) (11, 13, 15, 16). Both Domains contain an origin of replication that seems to start replication concurrently, although some Archaea contain multiple origins of replication that computer simulations have predicted are able of firing simultaneously (15). Having fixed origins of replication is something not commonly found in Eukaryotes (2, 12). Higher Eukaryotes can simultaneously copy DNA from thousands of starting points (11, 12).

Archaea and Bacteria also contain what are called plasmids, which are circular pieces of DNA that are not attached to the chromosome and contain genes that are advantageous to the organism (14). Archaeal plasmids have been shown to contain essential proteins that are involved in DNA replication (Orc1/Cdc6) (11).

Perhaps the most important advantage to sharing such similar DNA structure with Bacteria is the ability to share DNA (2, 14). Archaea and Bacteria are often found living symbiotically, sharing DNA that is advantageous for particular locations (2). This sharing of DNA is called Lateral Genetic Transfer and has been shown to be common amongst Archaea and Bacteria (14). Some Bacterial genomes have been shown to be comprised of up to 30% of material from Archaeal origin, with Halophiles and Methanogens being the most promiscuous (14) due to the large number of insertion sequences within their genomes.

These similarities in basic structure have lead some to hypothesize the possibility of Archaea sharing a closer ancestor with Bacteria rather than with Eukaryotes (11, 14) however, this is not a consensus view. This lack of consensus is due to the fact that while

the genome structure of Archaea is similar to Bacteria, its replication machinery is quite different and more similar to Eukarya.

Bacteria's replication starts with the recruitment of *trans* acting factors, called DNA-A, to the DNA-A box (11). DNA-A bends and melts DNA and then recruits the bacterial helicase (DNA-B), a homohexamer (11, 15). Bacteria recruit single strand binding proteins (SSBs), which are active as homotetramers, to bind the ssDNA to keep them separated and the site prepped for replication (11, 16).

Eukaryotic replication starts with a complex known as the origin recognition complex (ORC), a heterohexamer that recruits other pre-replication proteins, such as Cdc6 (11, 12). This completed complex recruits the minichromosome maintenance complex (MCM), a heterohexamer, and is thought to be the Eukaryotic helicase (11, 14, 15). Eukaryotes recruit replication protein A (a heterotrimer), which acts as a SSB protein holding DNA in its melted form for replication (11, 12).

Archaea replication begins with ORC/Cdc6-like proteins, genetic homologues of Eukaryotic regulatory genes (11, 15). However, Orc/Cdc6-like proteins are single proteins, not a homohexamer as is found in Eukaryotes (11) and show greater similarities in the frequency and location of DNA binding sites to the Bacterial DnaA (11, 15). These proteins recruit the Archaeal helicase, which is a homolog of the Eukaryotic MCM protein (11, 12, 15). It is made up of a either a homohexamer or double hexamer depending on the species (11). Archaea then recruits RPA, which acts as a SSB protein to hold DNA in its melted form for replication (11, 15). Although these proteins are not well studied, they seem to be closer in form to Eukaryotic RPA than the Bacterial SSBs (15).

This pre-replication process clearly shows the similarities in Archaea and Eukaryotes and the differences between Archaea and Bacteria. Archaea seem to have a simpler version of the Eukaryotic pre-replication mechanism that increased in complexity as Eukaryotes diverged from Archaea. This suggests that Archaea and Eukaryotes share a common ancestor, which is further solidified when comparing the DNA replication process.

All three domains start the actual process of DNA replication after the formation of the pre-replication complex. It is here that Bacteria most clearly distances itself with the other domains. The Bacterial primase, DnaG (a monomeric protein), creates oligonucleotide primers that recruit DNA polymerase III (16, 17). DNA polymerase III performs the DNA replication, forming a long Okazaki fragment (16-18). DNA Polymerase I then cleaves the primer and fills in the gaps, forming a newly synthesized DNA strand (16-18).

In Eukaryotes the primase creates an initial RNA primer and then DNA polymerase α creates a RNA-DNA primer hybrid (16, 17)This is read by DNA polymerase δ, which creates a short Okazaki fragment (17, 18). Dna polymerase δ finishes by removing the RNA-DNA primer (16, 17). DNA polymerase δ is then inhibited by RPA, whose presence recruits the DNA2 protein (17, 18). DNA2 cleaves the new stand, which is then cleaned up by FEN-1 and repaired in by DNA ligase (12, 17, 18).

Archaea go trough the same process as Eukaryotes; however, they do not use the same proteins. They do seem to have homologs of RPA, FEN-1 and Dna2 but they do not contain all the same polymerases as Eukaryotes (11, 17, 18). Archaea carry two different

families of polymerases that carry out the same function (11). Archaea in the Euryarchaeota phylum have both B and D polymerases (11, 17, 18). Polymerase family D seems to be distinct to Euryarchaeota and it is thought that this polymerases is primary function is proofreading (11, 14, 18). The phylum Crenarchaeota only carries polymerases in the family B (11, 14, 18). The Archaeal replication process seems to be more efficient than the Eukaryotic one as well. This is seen in the rate of elongation being closer to that of Bacteria while still going through a similar process of elongation to that of Eukaryotes (11, 14, 17, 18).

Transcription in Archaea is also similar to what is found in Eukaryotes. Archaea contain a single RNA Polymerase, which containing 11-12 subunits and closely resembles RNA Polymerase II found in Eukaryotes (2). Bacterial RNA polymerase is made up of only a few subunits (2). Archaea also have similar promoters to what is found in Eukaryotes as well as similar recognition sequences such as a TATA box (2).

Archaea's genetic structure and mechanism for information transfer show a clear split between the three Domains. Based on these processes one could hypothesize that Archaea and Eukarya share a closer common ancestor than does Archaea and Bacteria. Archaea are faced constantly with extreme conditions, which raises the potential risk of DNA damage. Their simple and efficient DNA replication and organization seems to give them an advantage in such conditions. The model of simple Eukaryotic-like machinery and organization built on top of Bacteria-like DNA seems to be the most adventitious for living in extremes. It is this model that has helped Archaea not only survive but thrive in extreme environments.

Classification of Archaea

Phylogenetically, Archaea are split between 5 phyla, with the two largest being the Crenarchaeota and the Euryarchaeota (2). At a functional and common level, Archaea are most commonly categorized into 2 distinct groupings made up of the following subgroupings: 1.) Environment-based (halophiles, psychrophiles / hyperthermophiles, and acidophiles/alkaphiles), 2.) Metabolism-based (methanogens and nitrifiers) (2, 3, 5, 19).

Metabolism

One of the ways in which Archaea are able to thrive in extreme conditions is by the unique metabolic processes they possess. Certain metabolic processes are unique in Archaea because their importance does not solely lie in gaining energy to use for growth and survival but also homeostatic maintenance. Being able to keep homeostasis is significant to Archaea, as the environments they are found in tend to be extreme. The stress of just being able to preform simple cellular processes in extreme environments has selected for Archaeal diversity specifically in unique metabolisms. Archaea are known to be able to preform chemoorganotrophic (energy from the oxidation of chemical bonds found in organic molecules) and/or chemolithotrophic (obtain energy from inorganic sources) metabolic processes (2, 5). Most Archaea, as well as Bacteria, are capable of chemoorganotrophic processes, and most are able to perform it anaerobically in some facet. There are also many Archaea that have been shown to be phototropic; however, this is usually to keep osmotic balance and only used as an anaerobic metabolic process when necessary (2, 5). Chemolithotrophic Archaea are distinct in the metabolic processes they use to both keep homeostasis and grow/reproduce (2, 5).

Chemolithophs

Chemolithophs are unique in their ability to obtain energy by the oxidation of inorganic compounds. This process is similar to chemoorganotrophic processes except for the use of an inorganic compound as the electron donor and sometimes as a reducing agent (2, 5). They also use an electron gradient to power ATP synthase to generate ATP. Most are also autotrophs that use CO_2 as their main source of carbon (2) These organisms typically have one inorganic source in which they oxidize and are named for this source; within the Archaea there are the methanogens and nitrifiers.

Nitrifiers Nitrification involves ammonia being oxidized to nitrite with the freed electrons being used to create an electron gradient that powers ATP synthase (2). For many years there were no recognized species of nitrification-able Archaea, instead there was only hypothesized nitrification-able Archaea, placed into the Phylum Crenarchaeota. Early studies showed that there was a large population (up to 40% of lower ocean microbes) of marine Crenarchaeota that they were capable of nitrogen fixation (2, 20). This was shown in ocean samples that contained ether linked lipids and unique ratios of ammonia to nitrite (2, 20). Recently, the first archaea from deep marine samples was isolated and gave rise to a new genus and species *Candidatus Nitrosopumilus maritimus* (20). This species has shown the ability to grow through aerobic oxidation of ammonia (thought to be the most common source of nitrogen for Archaea) and is the first of many marine archaea to be isolated (2, 20). There are marine bacteria that have been shown to also be capable of nitrification but Archaea have been shown to be able to do the process

with much lower resources (2, 4, 20). The significance of Archaea's ability to thrive and outcompete Bacteria in lower nutrient environments (no organic energy resources or sunlight) is because most of the ocean is a nutrient limited environment (2, 4, 20). This argues that Archaea, not Bacteria, is the major player in both marine environments and in the crucial marine nitrogen cycle. Recently, there has been a nitrification-able archaea isolated (*Nitrososphaera viennensis*) from multiple soil samples, which suggests that Archaea may also play a pivotal role in the nitrogen cycle of soil (21).

Methanogens Methanogenesis is the biological process of creating methane. This is primarily done through the reduction of CO_2 to H_2 to form methane (CH_4) although acetate can also be used (2, 4, 5). Methane is the byproduct in this reaction that creates an electron gradient to run ATP synthase, creating ATP. Archaea is the only known domain that can preform methanogenesis. This process occurs strictly under anaerobic conditions, and has been found across many extreme environments (5). The archaea responsible for the biogenic production of methane are diverse and well studied, fitting into five different orders that are commonly found in many environments. Methanogens fully depend on other organisms to break down complex molecules into CO_2 and H_2; they are almost always found along side Bacteria or other Archaea (4) Methanogens are extremely efficient at using CO_2 and have been found to outcompete all other cells in environments where CO_2 is the most abundant resource (4, 5). This ability to use only CO_2 and H_2 to survive in most environments shows again how resilient Archaea are in extremes. Being able to use very specific substrates and focusing on a singular metabolic process has proven to be a very advantageous strategy for Archaea (4). The prevalence of

methanogens across many environments has caused methane to be one of the key signatures looked for when studying seemingly uninhabitable environments and planets (2).

Environments

The main environments that Archaea are found in are areas featuring the following characteristics: extreme temperature, high salinity, and extreme pH (2, 22). Each of these environments presents numerous environmental obstacles (i.e. low oxygen, low nutrients) that other domains have trouble conquering.

Extreme Temperature

Temperature presents two different extremes, both high and low. Low temperature causes destruction of living tissue and membranes through the formation of ice crystals (2, 10). High temperature causes instability within macromolecules, especially proteins, leading to denaturation (2, 10). Thermophiles thrive in higher temperatures while psychrophiles live in lower temperatures. Archaeal species have shown the ability to thrive in both of these environments.

Thermophiles Thermophiles have an optimum temperature range 45° C - 80°C and hyperthermophiles have an optimum temperature above 80°C (2, 23). Archaea not only thrive in both these temperature zones but have also defined the highest temperature at which life is thought to survive (24). Recently, a cultured archaea was found to grow at 121°C, it was classified as Strain 121 and is a close relative of *Pyrodictium occultum* (24).

This is the highest temperature known to sustain biological life. With extreme high temperature, the goal is maintaining the structure of the membrane, proteins and genetic material. At high temperatures proteins run the risk of denaturing and membranes tend to become unstable. To over come these stresses, Archaea have evolved unique adaptations in both their protein and membrane. One of the key characteristics of a stable membrane is low permeability to solutes, or the ability to keep the environment out. Archaeal thermophiles have an unusually stable membrane mainly due to the use of ether linkages as opposed to ester linkages (2, 10). Ether linkages are more thermostable than ester linkages, which, allows the membrane to keep stability within a large temperature range. Thermophilic archaea have a 1:2 mix of archaeol to caldarchaeol lipids, which allows them to have a hybrid membrane that is both a bilayer and monolayer (10, 23) The caldarchaeol lipids are what form a monolayer and they contain many 5-6-carbon rings that can take up room. These rings squeeze the membrane and cause the membrane to tighten which is important in keeping the solubility low at high temperature (8, 10, 23). Thermophilic proteins have many adaptations to prevent the denaturation that normally happens at such hot temperatures. One of these adaptations is the reduction in size of their proteins by forming complex oligomers with more ionic bonds and a reduced flexibility (2, 25). This allows them to pack the hydrophobic core more tightly and reduce the contact of their hydrophobic regions to the heated solvent (2, 25, 26). Their proteins also have greater number of disulfide bonds and salt bridges, which create loop structures. (25, 26). They also place a charge on normally uncharged segments of the protein surface, which increases their stability (25, 26). This allows the proteins to be more rigid at higher temperatures and therefore resist the increased molecular motion that would threaten to

rip these proteins apart. Intracellular monovalent and divalent salts help increase the stability of their nucleotides (25, 26). It does this by forming a protective barrier around DNA through the attraction of the positive ions from the salts to the negative phosphate groups (25, 26). Some thermophiles also contain a higher ratio of G-C pairs within their DNA that is more thermostable, although this is not found in all thermophiles (22). While some of these adaptations are found in thermophilic members of other domains, it is their commonality and prevalence in Archaea that show its distinction from the other two domains. Thermophilic archaea are also a prime candidate for astrobiological research as they are often found around hydrothermal vents (2, 24). Hydrothermal vents were the first environment on Earth that do not have the sun as its primary energy source. Therefore, the activity at these locations is hypothesized to be the on of the best sources of energy for life to start on extraterrestrial bodies and has been hypothesized to help start life on Earth (27).

Psychrophiles Psychrophiles grow optimally at temperatures lower than 15°C and are unable to grow at temperatures above 20°C (2, 26). These conditions are found in over half of the ocean, which has an average temperature of 5°C and holds over 80% of the entire biosphere (2, 28). Lower temperatures affect cellular membranes by decreasing their fluidity and proteins by slowing enzymatic processes as well as protein folding (2, 26, 28). As a result, psychrophilic proteins usually have a less rigid core, which is hypothesized to increase the flexibility of these proteins. This is accomplished by increasing or decreasing the prevalence of specific residues, lowering the number of salt bridges, and altering the number of loop structures (2, 26, 28). Another characteristic of

cold-active proteins is an increased amount of alpha helix over beta sheets. This is hypothesized as alpha helixes are more flexible than beta sheets (2). The above changes allow the protein the ability to achieve a greater amount of molecular movement, which it needs to remain active in its native environment (29). This increased flexibility counteracts the effects of the Boltzman equation and allows cold-active enzymes to remain catalytic activity at rates that physics would otherwise predict were impossible (22, 26, 28). A more flexible protein usually contains a larger binding site while the catalytic residues are not changed (22, 26). Because the substrates are so slow in their movement, it is more advantageous for the protein to increase its catalytic activity by sacrificing some of its binding area (22, 26). Psychrophilic archaea are also thought to have chaperone proteins (e.g. cold-shock and heat-shock) that help proteins and RNA molecules maintain their active state, as colder temperatures cause these processes to become more difficult (2, 28). Psychrophilic archaeal membranes are unique in their lipid composition. As opposed to thermophiles that have higher caldarchaeol lipids compared to archaeol, psychrophiles have more archaeol (26, 28). This is change is hypothesized to be the cause of the increased permeability of necessary for the membranes of psychrophiles (28). While psychrophilic organisms make up a vast number of the total biomass of the planet, they are perhaps the least studied. This is especially true for psychrophilic Archaea as most research done with them is as a comparison to the other domains. This trend is changing however, as most planets found in the Solar System and beyond have been shown to have extremely cold environments (30). This has caused psychrophilic archaea to be another prime candidate for astrobiological research.

Extreme pH Another environment in which Archaea thrive is within the extremes of pH. They are found in both acidic and alkaline environments, both of which present major challenges for living systems.

Acidophiles Acidophiles are defined as anything that lives in a pH lower than 5.5 (2, 31). Areas that have such a low pH are often found at higher temperatures and so many acidophiles are also thermophiles. As a result, some of the adaptations for living in a low pH are dually beneficial for living in a higher temperature. The main pressure on organism living in acidic environments is the increased number of free-floating protons that creates a highly positive charged setting. This causes proteins to denature and membranes to disintegrate (2, 26, 31, 32). This causes surfaces of macromolecules to protonate, changing their charge and conflicts with the normally negatively charged internal cell environment (2, 26, 31). (Proton Motive Force?) To combat this acidophiles change their proteins' surface by adding more acidic (negative) residues to combat the positive environment (26, 31). Proteins of acidophiles also seem to use metal such as iron to help stabilize the enzymatic activities and some acidophilic proteins have been shown to be active at negative pH (31). The mechanism that allows this and the metals full purpose in it is still not known (31). Their membranes are very similar to thermophiles, a monolayer of ether linked caldarchaeol lipids containing multiple 5 and 6 carbon ring structures (31, 32). Ether linkages are more resistant to the lower pH than ester linkages and the monolayer membrane keeps ion solubility low with minimal energy expenditure (2, 31). Acidophiles also have to deal with keeping a higher internal pH to that of their surrounding. The internal pH of acidophiles is much lower than that of cells in a normal

environment but it is kept higher than its surroundings (2, 31, 32). This lower internal pH is kept by having an overall positive internal charge that is created by a membrane with low ion permeability and an increased amount of ion pumps (31, 32). The ion pumps drive protons out and potassium in to combat the external environments high proton content. This positive internal charge causes problems for protein folding and many acidophiles contain chaperone proteins to help in this process (31).

Alkaliphiles Alkaliphiles are characterized as organism that live in a pH of 8 or higher. Highly alkaline pools are also highly saline, making most alkaliphilies also halophiles. As a result, most adaptations in alkaliphilies are also advantageous for living/surviving in saline environments. Alkaliphiles are known to keep an internal pH as close to neutral as possible. This is done through the adaptation of a thicker membrane and numerous proton pumps to increase the intake of this scarce resource (2, 26). These organisms are perhaps, more properly labeled as polyextremophiles as they have adapted to high salinity and alkaline pH (22).

Halophilic Archaea Extreme halophiles live in environments from approximately 1.5 M (9%, w/v) to saturation (approximately 5.5 M for NaCl). The amount of salt for optimal growth is 3-5 M (20-30%, w/v) (2, 29, 31). The amount of salt in these environments causes membrane desiccation and causes protein destabilization/precipitation. In these environments the other main concern to the cell is maintaining osmotic homoeostasis. Normal cells lyse quickly/instantaneously in a hypersaline environment because their internal osmotic pressure is lower than their surroundings (33, 34). This causes water to

extrude from the cell at a rapid pace and the external salt ions to flow inward (33, 34). To combat this, halophiles use one of two mechanisms, either using organic solutes to combat the high external ionic strength (a.k.a. salting out) or by generating an intracellular environment that is similar to the external (a.k.a. salting in). Most Bacteria and Eukarya survive in saline environments by utilizing compatible solutes (osmolytes) (34-36). This mechanism results in a higher solute content in the cell, which keeps the external salt ions from entering via diffusion. While it is effective, it is inefficient as creating these molecules require both energy and materials (34-36). Archaea, however, selectively pump K^+ and Cl^- ions into the cell while extruding Na^+. This establishes an electrochemical gradient with Na^+ being kept out by the greater amount of internal K^+ ions (2, 34). Higher levels of K^+ cause the cell to have a higher internal positive potential than normal and this affects all macromolecules within the cell. In response, haloarchaea have adapted to their environment by increasing the number of acidic residues on the surface of their protein, which increases the negative charge on the protein surface allowing them to combat the increase in positive ions within the cell (26, 36, 37), and decreasing the number of hydrophobic residues thereby counteracting the increased hydrophobicity interactions with salt ions (26, 36, 37). Within this decrease of hydrophobicity is a decrease in the proteins size and reduction of its core. Normal proteins cannot fold in an environment with an increased salt content, but haloarchaeal proteins cannot fold with out it. This mechanism of salt-dependent folding is still not fully understood (26, 36, 37); however, one mechanisms that is thought to help is the use of eukaryotic like chaperone proteins (36). Another adaptation of the halophilic archaea to their environment is a diether-linked isoprenoid (archaeol) bilayer membrane (38-40).

This bilayer allows the halophilic membrane to have more flexibility when dealing with osmotic pressure. While bilayers are usually more permeable, halophiles account for this through large polar head groups, which are highly negative (38). This creates a negatively charged field that surrounds the exterior and the large size creates steric repulsion (41). This creates a membrane with low ion permeability while not costing the cell a lot of energy. Halophiles are unique in another way, through their use of phototrophic mechanisms. They utilize photosensitive proteins to keep an ionic balance and as an anaerobic energy source. Haloarchaea use two main pigments, Bacteriorhodopsin (export of H^+ for generating ATP) and Halorhodopsin (for excreting H^+ and importing Cl^-) (2, 37, 41, 42). Bacteriorhodopsin is a purple pigment that is up regulated when oxygen becomes scarce, which is often due to overcrowding. It gives halophilic archaea an additional means to create a proton gradient for the production of ATP. Halorhodopsin responds to the red/orange spectrum and regulates Cl^- intake, the main anion in halophilic archaea (42). Halophilic archaea have also been shown to be extremely resistant to heat, radiation, desiccation, and low oxygen environments (2, 37). As can be seen when comparing the different Archaeal niches, some stresses have multi-purposeful adaptations. The mechanisms behind haloarchaea's ability to resist all of these stresses are still unknown but it can be speculated that it is due to their adaptations being multidimensional. The adaptation of life in high salinity is deeply rooted in the tree of life. Hypersaline environments also extend water's ability to stay liquid at lower temperatures. Average temperatures found in the Universe are typically very cold, if liquid water is found on another planet it will most likely be saline. One can hypothesize that the halophilic

archaea's resistance to multiple stresses and ability to thrive in hypersaline environments makes them the best candidate for an astrobiological analogue.

Saline environments

Saline environments are defined as any environment in which the salt content exceeds ~2%. These environments are often subdivided into three categories: slight (2 to 5%), moderate (5 to 20 %), and extreme (20% to saturation) (2, 22, 37, 43). Hypersaline environments have a saturation point of around 35% (43). There are both dry (soil) and aqueous (lake) hypersaline environments although the research on highly saline soil based environments is extremely limited. As a point of reference, seawater is typically 3.5%, which places most of the microbes in the world's oceans under the categorization of slight halophiles. As a general rule, Eukaryotic organisms are found in high abundance in slight halophilic environments while Bacteria and Archaea are found in higher abundance in more saline environments. Archaea distinguish themselves as being the predominant organism as the salinity increases to hypersaline levels.

Hypersaline Environments

Most hypersaline environments have a salt concentration of at least 20% while many greatly exceed this. Some examples of these hypersaline environments include the natural environment such as the Dead Sea (33%) and the Great Salt Lake (27-30%) as well as man-made environments like artificial crystallizer salt ponds (37, 43). The higher salinity of these environments has given them the label of being "dead" environments, probably because of the low amount of life visible to the naked eye. A perfect example of

this is the ironically named Dead Sea, which has species from at least ten different genera of Archaea that thrive here (Oren 2012). Research on hypersaline environments is steadily growing; this exploration has shown that organisms living in these environments do not simply have to survive the high salt levels but many other conditions as well. Hypersaline environments tend to also have one or more of the following additional extremes: (i) high or low temperatures, (ii) high or low pH, intense solar/UV radiation, (iii) low water activity, and/or (iiii) low oxygen concentrations (1). These conditions are the result of a few geological characteristics that these environments commonly share. In hypersaline soil, such as the Atacama Desert, there is very little to no inflow of water. These desert environments are usually found around mountain ranges that block rainfall and have an impermeable layer below the surface usually in the form of dense clay (44). This creates a stagnant environment that has no turnover and vast areas that are above the saturation point of salt (44, 45). Most hypersaline lakes are endorheic, having no outlet to release water to the ocean (moss 1994) (44). In endorheic lakes, any water that reaches the lake will sit until it is eventually evaporated. This means any minerals that it contained will be left, leading to a steady increase in minerals and an exponential concentration of those minerals in the lake. These bodies of water have no recycling of water or nutrients, often having low oxygen concentrations and lower pH levels (46). This creates a stratified body of water that has levels with differing amounts of nutrients and oxygen. This is called a meromictic lake and they usually encompass a bottom layer that is hypersaline and microoxic (46) Concentration of hypersaline environments is further intensified when the rate of evaporation is higher than the rate of incoming water (precipitation, fresh water inlets). If the outflow of water (evaporation) is significantly

higher than water inflow (upwelling, precipitation, etc.), this can often lead to areas of dry salt crust, or salt flats. Salt flats (or salt pans) are the least studied of all hypersaline environments. They consist of giant evaporate fields that are composed of salts and more complex minerals. Common minerals and salts found in salt flats include: gypsum, quartz, KCl, halite (NaCl), and MgCl (47, 48). The evaporation process in both lakes and salt flats tends to be a slow tedious process that keeps salinity levels high and stable. This salinity is established on the origin of the initial water source and the surrounding environments make up.

Differing Ions

Hypersaline environments have different ionic compositions, and these can greatly affect the life that lives there. There are two different types of aqueous hypersaline environments that are defined by their origin, thalassohaline and athassohaline. Thalassohaline water originates with seawater that is isolated and its composition reflects this. The main salt of these environments is NaCl with other salts being in much lower abundance (44, 49). The Great Salt Lake is the most common example of this type of environment; Na^+ and Cl^- comprise 85% of its total ionic composition (37, 43, 49) This is nearly identical to seawater in percentage but it is much more concentrated. The northern arm of the Great Salt Lake has 105 g/L of Na^+ and 181 g/L of Cl^- in comparison to seawater, which has 11 g/L of Na^+ and 19 g/L of Cl^- (37, 43, 49) Athalassohaline waters do not originate from marine water and they are rich in different ions such as calcium and magnesium (44). The Dead Sea is the most common example of a athalassohaline lake; it has a higher concentration of Mg^{2+} and Ca^{2+}

(divalent cations) than Na^+ (monovalent cation) (37, 43, 49). Mg^{2+} and Ca^{2+} comprise 20% of its composition where as Na^+ only makes up 11% (37, 43)This ionic make up affects the organisms that live in these environments.

Life in Stable Salinity

Highly saline environments that do not show large fluctuations in salinity tend to be very low in microbial diversity. Living in a highly saline environment is extremely taxing on the organisms that live there, mainly due to the energy cost of osmotic homeostasis. These environments place great pressure on properly balancing intracellular and extracellular ions, which is an extreme selection pressure in these environments. This is especially true in hypersaline environments that do not fluctuate greatly in their salinity. Studies on hypersaline environments, while low in number, consistently show that as salinity increases microbial diversity decreases (46, 49-51). Salinity is an important selection pressure and is more powerful if this salinity is kept high. Environments that fluctuate in salinity, such as in areas that receive high annual rainfall or areas with constant inflow of fresh water, show a greater diversity of organisms (46, 49, 52). Differences in diversity are seen in the microbiome of meromictic lakes where the surface, which receives rainfall, is much more diverse than the hypersaline microoxic bottom sediment (46, 49). A recent study on the Dead Sea (53) has shown that there are small fresh water springs that flow a very minimal amount of fresh water from beneath its floor. Samples taken here in comparison to samples taken elsewhere on the hypersaline floor show huge spikes in diversity (53). This is thought to be completely different from the difference in salinity caused by the inflow of fresh water. The Dead Sea as a whole

has shown an increase in salinity the past 100 years (54). This increasing concentration of ions has had profound affects on the life there, as the overall diversity has greatly decreased (54). Diversity differences have also been seen in the Great Salt Lake, which has a huge difference in salinity between the North and South Arm (43). Its North Arm contains a higher percentage of Archaea while its South Arm contains more even numbers of all three domains (43). Higher stable salinity lowers diversity and this lower diversity is dominated by halophilic archaea.

Archaeal Supremacy

It has been shown that haloarchaea consistently dominate populations of microbes that live in the extremes of hypersaline environments. There are two reasons that have been generally accepted for this dominance of Archaea found in hypersaline environments versus environments with a diluted salinity:

1.) Only certain metabolic processes can function at these higher salinities

2.) Halotolerant organisms spend too much energy on homeostasis at higher salinity to be able to survive. Halophilic adaptations allow Archaea to thrive because of how efficiently they reach and keep homeostasis

Only certain metabolic processes have been found above certain levels of salinity, this includes phototrophic processes, nitrification, aerobic respiration (with differing electron acceptors), and methanogenesis (51, 52). Certain processes, like the ones aforementioned, seem to be the only ones efficient enough to meet the energy requirements of hypersaline life. Members within the class Halobacteria, which make up arguably the most resilient halophiles known, have been found to preform all of the

above metabolisms (2, 37, 51, 52, 55, 56). They are also the most common organisms found in extreme hypersaline environments, where they are usually the dominant microbes (2, 46, 51, 52, 55).

Halotolerant organisms keep homeostasis by excluding salt and making organic osmotic solutes to combat the ionic barrage. This is a very costly process and it only becomes more expensive as the salinity increases. Almost all Bacteria and Eukaryotes are halotolerant, very few share the any of the adaptations that halophilic Archaea posses (51, 52),). All members of Halobacteria are halophilic and possess a unique adaptation to hypersaline environments by actually bringing salt into their cell. Another unique adaptation halophiles possess is their much sturdier and less permeable cell membrane, that can stand up to the osmotic pressure. This allows them to keep out ions using a lot less energy to keep homeostasis (2, 36, 38).

These adaptations have allowed halophilic organisms to dominate the hypersaline environments they are found in. Most hypersaline environments have little to no water influx creating settings that are low in oxygen and nutrients. High salinity also lowers the freezing point of water, allowing liquid water to be found in places with an average temperature below 0C. Hypersaline environments differ in their ionic makeup, which can be as diverse as the life found there. These characteristics have made hypersaline environments prime candidates for astrobiological analogues.

Great Salt Lake

The Great Salt Lake, found in Northwestern Utah, is one of the most well-known aqueous hypersaline environments. It is also one of the largest saline terminal lake

ecosystems known. The main solutes found in the lake are as follows: Na, K, Ca, Mg, Cl, SO_4, HCO_3, CO_3, and SiO_2 (57). The lake is separated into two distinct arms, North and South, which differ greatly in their respective salinity. The Lake's main source of fresh water is by 3 rivers (Bear, Weber, Jordan) that make up 90% of the lakes water inflow (58). These all flow into the South Arm, which is much more diluted than the North Arm. The North Arm has no significant input from a fresh water source and receives most of its water from the slightly higher South Arm. This has lead the Northern Arm to become much more concentrated in salt than the South Arm. The South Arm salinity is around 9% where the North Arm's salinity is around 27% and can reach 30% (43, 58, 59). Much of the salinity comes from mineral rich hydrothermal vents that flow into the aforementioned rivers. (58) The North arm has a higher evaporation rate to water inflow, which has become even greater due to recent droughts (43, 60). This has greatly affected the rivers that feed the lake, whose inflow has dropped to one third of the amount compared to decade ago and one eighth of the amount compared to three decades ago (57). The South Arm's water levels have dropped as a result, which has lowered the amount of water the North Arm receives (54) This has caused the salt concentration to go up keeping the North Arm closer to saturation (35% NaCl). The Great Salt Lake also has a unique history that makes the study of the area and microbes with in it even more invaluable. The Great Salt Lake was once part of a larger lake named Lake Bonneville. This lake was created approximately 50,000 years ago when glacial runoff filled the Bonneville Basin, which reached into 3 states, including almost all of western Utah (61, 62). Around 40,000 years ago, the Basin breached a natural dam known as the Red Rock Pass and flooded out into the Snake River, carrying most of the basins water into the

Pacific Ocean (57, 61). This flood lasted about a year and took the Lake from 1500m to about 1200m, the current level of The Great Salt Lake (61, 62). During its peak, the Basin lost water at an astonishing rate of 1.15 million m^3/sec, and the event is credited as one of the largest geologic floods in the fossil record (57, 61). This greatly reduced the area of the lake to the size it is currently, and it lost both the main inlets and outlets that once fed Lake Bonneville. This resulted in the endorheic lake now known as the Great Salt Lake (61). The Great Salt Lake is not the only remnant remaining from the great flooding of the Bonneville Basin: 50 miles west of the Great Salt Lake is a saltpan known as the Bonneville Salt Flats. There are not many studies done on the life within Great Salt Lake, however the studies that have been done on its microbiome have shown a plethora of life. Studies on the South Arm have shown that the more saline surface (0-4m) contains much more overall Archaea, compared to the other domains, than the diluted bottom (8m) (43, 62). There is very little information on the microbiome of the North Arm but initial studies have shown that its waters and halite crystal edges are rich in Archaeal life (specifically Haloarchaea) (59, 63).

Bonneville Salt Flats

The Bonneville Salt Flats (BSF) is a giant saltpan in the northwest part of Utah. It is known for being the surface on which many land speed records have been set. It sits just west of the Great Salt Lake in the western part of the Great Salt Desert. It is 43 square miles and within it contains a span of 34 square miles span of perennial salt that is at least a foot deep (47, 64). This area was once part of the same water body as the Great Salt Lake, Lake Bonneville. That lake was drained 40,000 years ago creating a shallow water filled depression to the west of the Great Salt Lake, which quickly evaporated. This

was the genesis of the Great Salt Lake, the Great Salt Desert and the Bonneville Salt Flats (61). The BSF sits in a slight depression, the desert to its East is slightly higher and the desert to its West rises into the base of a small mountain range (47). This depression has created multiple aquifers that sit below the BSF and occasionally flood the flats. The salt that makes up most of BSF is five layered: 1) dense halite 2) fine grained gypsum 3) porous halite 4) fine grained gypsum 5) course halite (47). This five-layered stratum of salt sits on top of carbonate mud, clay, and a deeper lacustrine (lake) sediment layer (47, 61). The thick salt crust that makes up the BSF is mainly made up of salt that was from the original evaporation 40,000 years ago (47, 64). However the BSF receives annual precipitation (average of 4 inches in the winter months) that floods the flat and dissolves the halite surface. Some of this salt is discharged to lower levels and is carried out through a slow flow that happens in the underlying aquifers. Recent studies have shown that the salt crust is not becoming thinner, which indicates a recharging of salt (47). There are three aquifers that feed into the BSF and they are the main source of salt recharges to the BSF. The main source of salt is the shallow brine aquifer that sits directly below the flat. It is an extremely saline (172g/L) aquifer and it floods the flat during the winter months (47). The other two aquifers, an alluvial-fan aquifer to the west and deeper basin filled aquifer below, both contribute more diluted brine to the flats (47). This water floods the BSF and stays through the winter months until the summer months evaporate it away (47, 64). This creates a dynamic cycle in the salinity levels along the surface of the BSF. The BSF represents a unique environment in which to study life at its most extremes. Salt flats are the least studied of any hypersaline environment, which is likely do to the lack of visible life on its surface. The few studies that have been done on the microbiome show a

dominance of the *Halobacteriaceae* family and some Genera such as *Haloarcula* (65). Initial 16S rDNA gene sequencing techniques preformed in these locations have shown an Archaeal dominated in microbiome (66). There have also been some studies on the life found in fluid inclusions in halite crystals that surround hypersaline lakes (67). These studies have come against some debate as to the actual age of the halite but what is not argued is the presence of Archaea within the crystals. The BSF doesn't have any published studies on the life within. Life within the BSF would have to endure many environmental pressures that life on another planet would have to endure, including high salinity, low oxygen, and high radiation. Life, if found here, would have to adapt to a quick change, from freshwater to a dry hypersaline environment. This type of change is analogous to environments found within our solar system. The Bonneville Salt Flats is therefore an interesting candidate for an astrobiological analogue to study as a reference for future astrobiological missions.

Hypersaline Environments as Astrobiological Analogues

Hypersaline environments are mostly studied as astrobiological analogues, specifically Mars analogs due to their similar conditions: high radiation, aridity, and mineral rich composition (43, 45, 68). Hypersaline soils have a mineral composition that is extremely similar to the soil composition found on Mars, containing a lot of chlorate and perchlorate (43, 45, 68). The comparison with Mars is due to the fact that it is the only non-Earth soil we have physically analyzed. Mars' soil similarity to those found on Earth suggests that similar consistencies of soil may be found throughout the Solar System and beyond. While no physical samples have been taken, many satellites of our

neighboring planets show signs of liquid water, some with oceans beneath their icy surface (69). The conclusion that can be drawn from this is that intrasolar planet's hypersaline lakes found under many feet of ice (i.e. Europa) will have a similar composition to frozen hypersaline lakes found on Earth. In all, hypersaline environments seem to be a likely environment to be found throughout the Solar System. This supports the hypothesis that if life exists in Solar System, it is likely halophilic in nature.

Antarctic Hypersaline Environments

Antarctic environments contain a unique hypersaline environment, one that deals with extremes cold rather than extreme heat. This is because salt water has a much lower freezing point than pure water due to its impurity (salt), preventing the formation of hydrogen bonds needed in order to make ice (2). There is a direct correlation between the salt concentration of water and its freezing point, with hypersaline solutions having extremely low freezing points. The surface lakes of Antarctica are formed when ocean water is isolated and condensed.

Don Juan Pond The lakes found in Antarctica differ in both salt concentrations and temperatures. At the most extreme is Don Juan Pond in eastern Antarctica, which is the saltiest body of water ever recorded on Earth. It has a salt concentration nearly 18 times that of seawater (40% by mass) and can reach temperatures of -50 C (30, 70). Most of its salt is made up of $CaCl_2$ and it is thought to replenish its water through a deep aquifer upwelling and a slow flow from evaporating permafrost (30, 70). This slow surface flow is extremely similar to what is thought to happen on Mars (70), 16) This hostel

environment and its water flow is possibly an analog to water that has been found to condense and wash down Martian slopes (71).

Lake Vostok The largest lake in Antarctica is lake Vostok, which is located in the slightly east of its center. It has been covered by ice for around 15 million years and the ice layer is somewhere around 4,000 m thick (72). The lake is separated into a northern and southern arm, with the southern arm having less ice cover. Due to having less ice coverage, the southern arm is deeper and is less saline than the northern arm (73). The southern arm was recently drilled and sampled, showing an abundance of life. The initial characterization showed that it is mainly Bacteria that inhabit Vostok with Eukarya and Archaea at lower numbers (72). This is to be expected as the southern arm is much less saline than the northern arm and seems to be the main port for glacial water circulation (73). Further studies will be aimed at the deeper northern arm, which has a highly saline sediment (72, 73).

Deep Lake Another hypersaline lake within Antarctica is Deep Lake, which is known for the isolation of *Halorubrum lacusprofundi,* the first haloarchaea isolated from a cold environment (56). It has a temperature range of -40°C to -20°C and a salt concentration of around 30% (56, 74, 75). It is a marine derived lake that was isolated 3.5 million years ago (74). Archaea dominate Deep Lake, in fact the initial studies that isolated *H. lacusprofundi* were unable to grow any bacteria in the 100% deep lake medium (46). There have been bacteria found in the lake but only in the summer, when the lake is slightly saturated (46, 74) The ecology of Deep lake is primarily made up by 4 genera of

Archaea that make up 72% of the lakes microbiome (74). Deep Lake has recently came under more interest due to recent studies pertaining to Archaeal biofilms (74). It is believed that the biofilm creates a medium in which it is easier to share genetic material. The 4 genera in deep lake share their genetic information, not just between species but also between genera (74). It has been found that the most commonly shared genetic information were those pertaining to metabolism. They were shown to share sequences up to 35,000 base pairs long (74). This genomic partnership was increased in organisms found to be sharing a biofilm, which infers a possible reason for an Archaeal biofilm (74). This noncompetitive genetic interaction shows how Archaeal communities are uniquely adapted to not only survive in extreme environments but to thrive in them. This is not the only place where Archaea are found to utilize biofilms, and this has opened up a new and exciting avenue for further Archaeal study.

Atacama Desert

The Atacama Desert is on the Western coast of South America and contains the driest, oldest habitat that has been found on Earth. (76) It is one of the only hypersaline soils to be studied for a microbial presence. The Atacama Desert is unique in not having extreme temperatures (37 °C max high, -5°C max low) in comparison Death Valley's temperatures, which can reach 50°C for many days in a row (45, 76). Its dryness comes from its geography (height and positioning to surrounding mountain) and its lack of precipitation. A survey of the desert, taken between 1994 and 1998, showed only had 1 true rain event that measured 2.8mm (76). The area had almost no cloud cover and a strong constant wind from its Pacific coast. The desert as a whole has no significant

source of water, this includes in the form of groundwater, due to a deep layer of impenetrable clay, and in measurable runoff from the surrounding mountain ranges, with the exception of a few eastern spots (44, 45, 76). All of these factors together have caused the dryness found in the Atacama Desert and, over the years, has caused it to become the driest environment found on Earth. The Atacama Desert spans 1,000km and its makeup is highly variable through out. There are extremely small and shallow thalassohaline lakes on the eastern side of the desert, which receive water from volcanic rock and a small eastern mountain range (44). Atacama's soil is mineral rich and contains salts fields, made up of many different salts such as: sodium chloride, magnesium chloride, chlorate, and perchlorate (45, 68). It is also contains nitrate fields, which are thought to be of atmospheric origin and are common due to the arid nature of the dessert (76). Many of the earlier studies done on the Atacama desert had very little success in finding life, perhaps due to the shallowness and specificity of their digs (45, 76). More recent studies have looked deeper, taking samples 5m below the deserts surface (45). They found that the samples were teeming with microbial life from 2m to 5m deep. This encompassed both Bacteria and Archaea, including the newly proposed genus of halophilic archaea named Halomicrobium (45, 77). This genus is made up of halophilic archaea that also oxidize nitrogen (nitrate) (44, 77). This is not the only study to find Archaea, specifically Halobacteriaceae, in the Atacama Desert (44, 77). Characterizations through out the desert show that the microbial community is completely dependent on the mineral composition and concentration of specific areas. Salinity is highly variable within the desert, with some spots having levels as low as 1% and in others as high as 35% (44, 45). A recent study found that Archaea were exclusively located in areas with a higher salinity,

as all the species found were identified as belonging to the family Halobacteriaceae (44). In the areas that Archaea were found, they were the dominant organisms usually taking up the majority of the specific microbiome (45)12). It has also been shown that Archaea tend to be the most prevalent organism on the surface (44). Life found here is pushed heavily toward many different stresses including desiccation, salinity and UV radiation. Finding life in this environment has furthered the hope of finding life on another planet. One of these other planets is Mars whose soil composition taken form the Phoenix lander has shown many similarities to Atacama's (45, 68). Most importantly is the high amount of perchlorate and chlorate found in the Atacama, which are oxidizing agents that are normally thought of as life hindering (45, 68). It has recently been shown (45) that haloarchaea can grow in high perchlorate media and can actually use it as a electron acceptor in anaerobic metabolism (45). With the success of Curiosity and a future mission to Mars already being planned, the Atacama Desert seems like the best candidate to test vehicles that will look for biomarkers on the Martian surface.

Astrobiology

Astrobiology is the study of and search for life in the Universe. The first assumption that we have about life in our Universe is that it requires liquid, specifically liquid water. Although there are planets and satellites within our Solar System that have makeups of other types of liquid (methane, ethane), the most interesting astrobiological candidates are those that have liquid water on or within their surface (78). The requirement of liquid water is the most agreed upon requirement among almost all

astrobiologist (27, 79). From here, theories range greatly concerning how extraterrestrial life could possibly differ from Earth-life. These hypotheticals include life using different energy sources and being composed of non-carbon building blocks (80). There is only one example of life that we know, and that is life on planet Earth. It is the only life we know to exist within the Universe, and so the study of how it came to be is important to understand what to look for in candidate planets elsewhere.

Life's Emergence

The assumption that life is an emergent property and not unique to Earth, is built upon the current general understanding of how life arose and evolved on Earth. The origin of life had two phases, the abiotic build up (environment), and the actual development of a living organism (27, 81). The abiotic phase involved the accumulation of three crucial elements: liquid water, energy sources, and a variety of chemical building blocks.

Abiotic phase Water is the universal solvent, and its presence appears to be a crucial environmental condition for all of life. This widespread use of water makes it a crucial part of the abiotic environment. Liquid water appears to have formed on Earth around 4.4 billon years ago, through a cooling Earth that condensed gaseous H_2O and experienced constant collisions with meteorites and comets (27, 82). The early Earth also had an abundance of free energy sources, each with varying importance and amount available. The major sources seem to have been solar radiation, electrical discharges, volcanoes, shockwaves, and radioactivity (27, 83). Solar radiation (ultraviolent light) was the most abundant energy source on early Earth, but it is detrimental to the creation of

macromolecules (27, 81). Most widely accepted experiments use electrical discharges as their source of early Earth's energy (27, 79).

The creation of simple monomers (ammonia, carbon monoxide, aldehydes, etc.), more complex monomers (amino acids, sugars, lipids, etc.), and polymers (proteins, DNA, RNA, etc.) gave rise to the environment from which life emerged (27, 79, 83). This has become recognized as the primordial soup theory (or Oparin-Haldane Hypothesis) and is accepted by the vast majority of scientists in the origins of life field (84). This "soup" took a long time to create and involved a long period of chemical abiotic synthesis of organic compounds (27, 81). The library of theories involving the creation of these biomolecules is vast, disputed, and incomplete. Urey-Miller's experiment is the most famous example of early biomolecule creation; it showed the creation of amino acids, lipids, sugars and other biomolecules under early Earth-like atmospheric conditions continuously exposed to electrical discharges (27). Experiments like this have shown that it is possible to create most of the molecules that are thought to have been crucial for the environment from which life can arise. The key molecules synthesized in these experiments are amino acids, nucleotides, sugars, and coenzymes (27, 81, 83).

The last question relating to the abiotic phase is: how did these molecules come together in such a vast environment? The answer seems to be condensation. The primordial soup, from which life emerged, probably was found in an evaporating pond or on a selective surface like a polarized mineral (27, 79, 84).

The primordial soup did not at first produce a living cell, but more likely a proto-cell that evolved into a living cell (27). This proto-cell was unique in that it emerged the

complexity of organization from unorganized molecules. It most likely had a permeable membrane made from amphiphilic molecules that kept water out (27, 79, 82). It then obtained a replicating molecule similar to DNA or RNA (27, 85). RNA, or an RNA-like molecule, is the established choice due to RNA's catalytic properties as well as its importance in biosynthesis processes (27, 82, 85).

This RNA proto-cell most likely led to further proto-cell that obtained proteins and a system where the RNA coded for these proteins (79, 82). This part of the story is still the most unknown and clouded aspect of the origin of life. What is known is that when this relationship between RNA and protein solidified, cells started to metabolize and life quickly flourished.

Biotic phase When this proto-cell evolved into something that could replicate consistently and metabolize it became a "living" entity. The word life is a complicated and not easily defined word to use, but it is known that the use of this word requires a few key attributes. All living things that have been found so far metabolize, replicate, and undergo Darwinian evolution (79, 82). There is believed to be at least two separate splitting events from out last common ancestor to the 3 separate Domains as we define them today (2, 27, 79, 82). The first split is estimated to have happened around 3.7 billion years ago, about 300 million years after the first living cell (2, 82). The general consensus is that after the first replicating cell emerged; it faced differing environmental pressures that caused it to split. This split resulted in a Bacterial-like organism and an Archaeal-like organism. Eukaryotes do not seem to have emerged for at least another 1.5 billion years after an endosymbiotic event that involved the merging of a Bacterial cell

and an Archaeal cell (2, 79). Evolution continued its course and complexity continued to emerge in the form of complex cell, structures, and body plans (27, 85) Niches and differing selective pressures caused a massive split into separate kingdoms and species.

Emergence is the vital step in both the origin of life and its diversification into three different domains. Emergence of complex patterns from individual agents is something seen throughout nature; from spiral galaxies to sand dunes (86). As seen in the outline of the origin of life, basic building blocks are assembled and then more complex structures form. This pattern of building complexity continued from the emergence of living single-celled microbes to multicellular creatures. Cells then formed tissues, which formed organs and eventually bodies. Each part had a function more complex than the last, resulting in a continuous gain in complexity on top of existing simple agents (79). A remnant of this process is seen in the basic code and biochemical pathways that most complex life still shares with simpler organisms (79, 82).

This theory of life's emergence has big implications for astrobiological research. Life as an emergent property, means that it's not only possible for life to exist elsewhere in the Universe, but it is more likely than not. Another planet simply needs to meet these starting conditions for life to emerge. If life is inevitable with these conditions, then the discovery of life on other planets comes down to finding a planet that has, at some time, met the correct conditions. The estimated number of planets is growing as our telescopes reach farther and farther into the Universe. This number makes the probability of there being a planet with the right conditions for life much more likely. Furthermore, a planet only needs to have these conditions present for a certain time period to allow life to emerge. As Earth has shown, living organisms find a way to live in habitats that would

seem uninhabitable from the outside. Although the environment for life to start is fairly specific, life is unbelievably resistant once it is achieved and evolution takes its course. Of all life forms studied, Archaea have been shown to be the most resistant to extreme environmental conditions, even space (80). Archaea also appear very early in the evolution of life, an important distinction if a planet only meets favorable conditions for a short time. If life emerged on another planet and still lives, it is likely that it will be similar to something found within the Domain Archaea. As seen with convergent evolution found in different places on Earth, if two organisms have similar pressures they often evolve in the same way. Other planets tend to have much more hostile environments than what is normally found here on Earth. This seems to suggest that Archaea are the best candidates as an analog to study for astrobiology. An in-depth study of Archaea's ability to live in different hostile environments would be another crucial step to understanding life in the Cosmos.

Habitable Planets

In looking for habitable planets there are two different candidates, intrasolar planets and satellites and extrasolar planets and satellites. Initial research into the intrasolar planets and satellites has indicated that Mars is the best candidate in our solar system of a celestial body that could have harbored life at one time. While Mars seems to be the best candidate, there are other candidates in our solar system that could possibly harbor life. Intrasolar astrobiological research is still in its infancy but it is growing due to new technology and a growing public interest. It is pushing past the solar system, into the surrounding galaxy and out to the far reaches of the Universe.

Mars

The thought of there being life on Mars relies on a few hypotheses about what early Mars was like. Mars appears to have had liquid water on its surface and an atmosphere that was as thick as Earths. It also appears that it had an early Earth-like surface much earlier than Earth did (71, 87). If it had conditions that were conducive to life's emergence, then perhaps life originated there. The discovery of water on Mars has been validated through numerous missions to the surface and with satellite photos (71, 87, 88). It is clear that water forms and perhaps slowly flows on the slopes of Martian hills as the planet changes seasons and the temperature warms. This also is seen at the polar caps, where there are zones that reach warmer temperatures to that needed for liquid water (71, 87, 89, 90). While there is evidence of liquid water, there is not yet an answer of how much. What is certain is that there are soils and salt flats, similar to Earth (68, 71, 88, 90). NASA's Curiosity rover, currently sitting on the surface of Mars, is continuously showing new evidence of a wet Martian past (88). Martian meteorites and soil samples taken by other Mars Rovers have also shown that Mars seems to have had a wet past (91). The most recent NASA mission, MAVEN, is studying the current atmosphere of Mars and is revealing clues to its past atmospheric conditions (90). If it can show that Mars had an atmosphere similar to early Earths, and for a long period of time, more support will be given to the hypothesis that life possibly emerged there (43). These rovers and satellites have shown that while Mars had a wet past, the presence of water disappeared and did so fairly quickly (43, 88, 91). This means that if life did emerge on Mars it faced an aqueous environment that was most likely concentrated in substrates. This is very similar to both

the Great Salt Lake and Bonneville Salt Flats, both of which were quickly concentrated. Current Martian missions have shown that the Martian soil is very similar to hypersaline deserts found on earth (37, 43, 71, 88). The Atacama Desert's soil appears to be the most similar, as it contains similar levels of perchlorate (76). While this is normally a hindrance to living organisms, recent studies have shown that halophilic archaea can live in these soils and even use chlorate as an electron acceptor (68). The most important future question for Mars is about its past. Current Martian missions have shown that Mars had a wet past but the next question to answer is for how long. Halophilic Archaea have been shown to have a high tolerance to the extremes that would be needed to live currently on or beneath the Martian surface (63, 80, 91). The environments haloarchaea are found in, hypersaline lakes and soil, seem to be what was once, and may still be, most prevalent on the Martian surface.

Europa

Europa is a similar size to our moon and contains what seems to be a liquid ocean within its icy outer core. Its surface is extremely cold and contains giant cracks that stretch all along its icy surface (92, 93). This points to possible plate tectonics, and more importantly, it points to Europa being pushed and pulled by Jupiter's gravitational pull (93). This pushing and pulling causes the planet to flex, resulting in kinetic energy that might heat its core. This heat could potentially be released through volcanoes and cracks in its inner mantle. This would result in a possible energy source, and more importantly, could result in the ocean having pockets that are Earth-like in temperature (92, 93). This hot inner core is also supported by Europa's strong magnetic field (92). Satellites that

have studied Europa have shown that its ice core averages 10km in depth, but there seems to be pockets of thinner ice (94). Once again, this points to an internal heating that has raised the temperature of the ice enough to make it liquid. New spectrometer readings from W. M. Keck Observatory show that Europa may contain a salty liquid water ocean of ~100km in depth (two to three times the volume of all the liquid water on Earth) (69)

This ocean may have a different composition from Earths ocean, but it has both a liquid ocean and the possibility of free energy in the form of deep sea vents. This leads some to believe that there might be life teeming beneath Europa's surface (92).

Titan

Titan's geological activity is more debated than that found on Europa. Images show what seem to be icy volcanoes that shoot plumes of methane skyward, forming Titan's thick nitrogen-methane atmosphere (78, 94). Some argue that while this is true, the volcanoes seem to be dwindling, and the methane spewing is the end of Titan's geologic activity (39,). New images from the Cassini satellite have shown Titan to have massive surface lakes made of liquid methane and ethanol (78, 94). These conditions seem not very conducive to support the emergence of life, but they do show that Titan has a history of geologic activity. Cassini has also found massive salt flats, which could hold halophile-like organisms, if life does persist there (78, 94). Titan also has a thick icy core averaging 40km in depth, but it does exhibit liquid at the surface (78, 94).

Extrasolar Candidates

When looking at sheer numbers, it seems likely that life could exist on Exosolar planets that show similarities to Earth's composition. However, these planets are out of reach by todays current travel technology. We can prepare for future voyages to Exosolar planets by studying life on Earth and grasping a better understanding of its emergence. The preparation as to what to look for in these extreme environments can start here on Earth, in places where conditions seem to be similar.

While there is interest in the total amount of planets existing within the universe, the interest to astrobiology is in regards to how many are Earth-like. The major current study into these Earth-like exoplanets is a NASA funded Kepler mission. NASA outlines the Kepler mission as to "determine the frequency of Earth-size planets in and near the habitable zone (HZ) of solar-type stars" (95). This mission has started a database where future findings can be stored like genetic information in the NCBI database. This database is called the Multi-Mission Archive at the Space Telescope Science Institute (MAST) and already holds information on over 152,919 stars (95). The Kepler telescope looks for stars that are the agreed upon as "appropriate" in size, and then stays on these stars for a set period of time. The information taken up during this time is then uploaded to MAST, where astronomers can analyze the data, looking for Earth-like planets (95). This aforementioned habitable zone is where a planet needs to be, in distance from its star, to contain liquid water. Liquid water has become the most important ingredient when looking for planets that could potentially accommodate life (96, 97). The most current number released from the Kepler mission team's analysis of MAST shows more than 3500 planet candidates with 100 being in a habitable zone (96). This number is impressive because it does not include the entire database of MAST, which in total makes

up less than 1% of the stars thought to inhabit the Milky Way (96). What is even more extraordinary is that the false positives are only 5-10% of the deemed planet candidates (96, 97). This shows that the number of Earth-like planets is numerous, which makes life's emergence on another planet a much more probable event. This mission does not include other planets that may have at one time been within a habitable zone or planets that will eventually reach this zone. If the probability of life's emergence can be calculated and predicted, then these currently inhabitable planets may be the next subjects of future missions. The numbers are promising and are continuously growing. These exosolar planets seem to be a great starting point for the search of other life; however, their distance from Earth is astronomically far. Our closet exosolar planet that we currently know of is 4 light years away (60). A journey to Mars takes about 500 days on current technology, and Mars is our closest neighboring planet (98). While we are a long way from traveling to theses exosolar planets, the number that could theoretically harbor life is great.

Conclusion

The one thing we know about life is that is that it is resilient. Nature and scientists have pushed carbon-based life on Earth to many extremes and have proven its ability to withstand some of the harshest conditions that the Universe can present. With this said, the origin of life on Earth is still an uncertain subject that lacks a definitive theory as to how the events unfolded. The origin of life on Earth has many competing theories, each with its own compelling data and similar main ideas. Over the past few decades, general scaffolding has been created based on the likelihood of events and lab experiments recreating early Earth conditions. If the origin of life on Earth is not unique, but instead

an emergent property, then it's not a wild assumption to suppose that similar life could exist within the Universe and the Solar System. This idea of life as an emergent property heightens the probability of its existence elsewhere in the Cosmos. While a planet must meet some key requirements for life to emerge, once it does and Darwinian evolution takes its course, life will predictably survive. This allows us to take into consideration planets that are deemed currently inhabitable (Mars) as potential astrobiological candidates. If these planets had life at one time, it is very possible that they could still have remnants remaining. This leads one to inquire about the types of environments that are likely to harbor this life and if they are common in the Solar System. Missions like the Phoenix Lander on Mars and the Cassini Mission to Saturn show that liquid water is common in our own Solar System, and this water has a high salinity (68, 87). If life is to be found in the Solar System or Universe, then it is likely to be found in a hypersaline environment. That postulation makes life found in hypersaline environments on Earth (like the Bonneville Salt Flats) a good candidate as an astrobiological analogue.

Objectives

This study examined and characterized the previously unknown life that exists in the Bonneville Salt Flats. The objectives of this thesis were 1) to show that life, specifically Halophilic Archaea, exists within in the Bonneville Salt Flats (BSF) 2) to link each isolate to its native niche in the BSF through phylogenetic analysis to examine how environmental pressures affect the diversity of genera in a range of microenvironments and 3) to analyze the effect micro-environmental forces (setting) have on specific phenotypes (growth and pigment presence) of the different isolates.

The hypotheses tested in this chapter were 1) that Haloarchaea exists within the Bonneville Salt Flats, which was previously assumed to be lifeless 2) that microenvironmental pressures affect the genera diversity of haloarchaea within the BSF 3) environmental pressures from differing settings within the Bonneville Salt Flats have a direct impact on certain continuous phenotypes of the cultured isolates.

To address the first hypothesis, samples from the BSF were cultured and isolated, with five isolates randomly selected from six different microenvironments. The isolates' 16s rRNA genetic sequence was amplified and sequenced. To address the second hypothesis different phenotypes were measured (growth curves, presence and density of bacterioruberin, morphology) and phylogenetic trees constructed using the sequenced 16s rRNA genetic material of the selected isolates. To assess phylogenetic relationships among these cultured strains, phylogenies were reconstructed using a Bayesian approach, with and without a relaxed molecular clock model, and using a maximum likelihood approach. The phylogeny obtained with a relaxed molecular clock was used to test whether phenotypes were significantly correlated with genotype, using Arbor software to run the functions fitDiscrete and fitContinuous. The third hypothesis was addressed by testing whether specific microenvironments significantly impacted phenotypic variables (e.g., growth and pigment presence) by implementing analysis of variance in R.

CHAPTER 2

METHODS

Initial Isolations

Samples of the salt crust itself were collected from seven different locations and depths (smooth surface, crack, upwelling, large upwelling, large upwelling deep, UPI 2nd Layer) in the Bonneville Salt Flats on 8/8/2012. Samples were collected with sterile tools and placed into sealed, sterile containers, which were then shipped to the lab. Once in the lab, five grams of each sample was added to forty milliliters of media in an Erlenmeyer flask to dissolve the salt crystals. This process was accomplished at room temperature (25°C) without shaking in order to reduce the possibility of excess free radicle formation that would kill the cells. Once dissolved, two hundred microliters was aliquoted and spread onto two plates of the same media. This process was repeated for two different types of media: Modified Growth Media (MGM), which contains 23 % (~ 4 M) salt water (NaCl, MgCl2, MgSO4 KCl, pH 7.5) and five grams Oxoid peptone and one gram of yeast extract per liter (pH 7.5), and R2A modified for halophiles (SMR2A), which is R2A at 10 % strength supplemented with NaCl and magnesium (99). Plates were incubated at 37 and 42°C for seven days and checked daily for growth. After a week of growth, the plates were removed and three representative colonies of each type were picked. The resultant isolated colonies were then re-streaked until isolation. Each re-

streaked colony was incubated for up to seven days at the temperature it was originally isolated.

Plate-based Growth Curve Studies

All thirty isolates studied were individually streaked onto Modified Growth Media (MGM). Representative colonies from each were picked and used to inoculate separate cultures (five mL) of MGM liquid media. The cultures were incubated for seven days at 40°C at 200 rpm then 100 uL, per plate, was pipetted onto five MGM plates. Each plate was incubated at a different temperature (10°C, 20°C, 30°C, 40°C, 50°C) and checked every 12 hours until growth was observed.

Culture-based Growth Curve Studies

All thirty isolates studied were individually streaked onto MGM. Representative colonies from each were picked and used to inoculate separate cultures (5 mL) of MGM liquid media. Cultures were incubated for five days at 42°C at 200 rpm after which, two mL of the culture was used to inoculate 20 mL of MGM in a Klett Flask. The resulting cultures were incubated at the optimal temperature (40°C) at 200 rpm. The cultures were checked regularly and measurements of turbidity taken with a Klett Summerson Photoelectric Colorimeter until stationary phase was reached.

Pigment Extraction and Measurement

Membrane associated pigments were extracted from all thirty isolates examined. Each organism was grown until late log phase ($OD_{600} > 1.5$) as described for the growth

curve studies except that the culture volume was 25 mL. The culture was then divided in half and the cells pelleted by centrifugation (14000 rpm for 10 minutes). After the supernatant was aspirated, the both pellets were re-suspended with one mL of molecular biology grade water and then centrifuged at 14,000 rpm for 30 minutes. The supernatant was again aspirated and one mL of 100% acetone was added to one tube and 1 mL of a chloroform/methanol (2:1) mixture was added to the other. Both tubes were vortexed using a Fisher Scientific Touch Mixer at 2800 rpm for five minutes to solubilize the pellets and then subjected to centrifugation (14000 rpm for 20 min). The pigment rich supernatant was collected and its spectral signature analyzed from 300 to 700 nm using a Shimadzu UV-160 spectrophotometer. (100)

Genomic DNA Collection

All thirty isolates examined were grown as detailed in the growth curve studies (five mL cultures). Cultures were incubated for seven days at 40°C at 200 rpm and then two milliliters of each was centrifuged (14,000 rpm for two minutes). Genomic DNA was isolated by first re-suspending the pelleted cells in basal salts solution (250 grams NaCl, 20 grams $MgSO_4$, three grams $Na_3C_6H_5O_7$, 2 grams KCl per liter). The solubilized pellets were then lysed with a sodium hydroxide-SDS solution (0.2 M and 1% respectively) for less than 3 minutes, and neutralized with dilute acetic acid. The resulting cellular debris was then centrifuged (14,000 rpm for 15 minutes). Nucleic acids were then precipitated by mixing the supernatant with a 100% ethanol and sodium acetate mixture and incubating them at -20°C for 12-16 hours. The genomic DNA was then pelleted by centrifugation (14,000 rpm for 20 minutes), and subjected to two washes with

70% ethanol with centrifugation steps in between. The resulting pellets were then dried via a rotary evaporator and re-suspended in 50 μ L of molecular biology grade water.

PCR Amplification

A portion of the 16s rRNA subunit was amplified using the Archaeal oligonucleotide primers ARCH2F (TTCCGGTTGATCCTGCCGGA) and Arch1509R (GGCTACCTTGTTACGACTT) in polymerase chain reactions (PCR), yielding an approximately 1400 base pair fragment (101). The total PCR reaction volume was 50 µL, including 50 pmol of each primer, 0.2 mM of each dNTP, 1x MasterTaq PCR Buffer (Eppendorf, Westbury, NY), 1x TaqMaster additive (Eppendorf, Westbury, NY), and two units of MasterTaq Enzyme. The thermocycler program included a denaturing time of five minutes at 95 C, followed by 30 cycles of one minute at 95 °C, one minute at 50 °C, four minutes at 72 °C, followed by a final extension time of five minutes at 72 °C. PCR products were electrophoresed at 60 V for 45 minutes on a 1.5% high melting temperature agarose gel with a 10,000 bp ladder to examine the quality and quantity of each reaction.

Cloning and Sequencing of DNA

PCR products for each strain were sequenced at the University of Alabama at Birmingham Center for AIDS Research Sequencing Core (CFAR) using Archaeal primers: ARCH2F (TTCCGGTTGATCCTGCCGGA), ARCH1509R (GGCTACCTTGTTACGACTT), ARCH330F(CCGGGCCCTACGGGG), ARCH1368R (GACGGGCGGTGTGTGDA), ARCH929R (TCCGGCGTTGAGTCCAATT),

ARCH646R(GGATTTCACTCCTACCCC) (101). Forward and reverse sequences were assembled using CodonCode Aligner version 4.2.3 (CodonCode Corporation, www.codoncode.com) to generate a consensus sequence for each clone.

Two sequences had to be further amplified using a cloning protocol to obtain a higher quality sequence read. To clone DNA sequences from PCR amplifications, cleaned PCR products were ligated to a plasmid vector using the Promega pGEM-T Easy Vector System II (Promega, Madison, WI), following the manufacturer's instructions: one µL of each PCR product incubated with five µL of 2X ligation buffer, one µL of pGEM-T Easy Vector, two µL of PCR water, and one µL of T4 ligase (10 µL total reaction volume). Ligation reactions were incubated for 16 hours at 14 °C. Following incubation, each ligation was transformed into *E. coli* JM109 competent cells (JM109; Promega, Madison, WI) by adding two µL of each ligation reaction to 50 µL of competent cells. Transformation reactions were incubated at 0 °C for 20 minutes, heat shocked for one minute at 42 °C, incubated at 0 °C for 2 minutes, then incubated with 900 µL of complete Super Optimal Broth + glucose (SOC) media at 37 °C for 1.5 hours at 150 rpm. 100 µL and 50 µL of each reaction were plated (Lysogeny Broth containing 28 ul / ml ampicillin (LB-amp)) with 100 uL of isopropyl β-D-1-thiogalactophyanoside (IPTG) and 20 µL of 5-bromo-4- chloro-3-indolyl-β-D-galactopytanoside (XGAL) added. Plates were incubated at 37 °C for 24 hours. After incubation, plates were screened for colonies containing plasmid vectors with the appropriate insert, indicated by the presence of a white colony. Positive colonies were then removed from the plates using sterile toothpicks, added to 1 mL of LB-amp liquid media, and incubated at 37 °C and 150 rpm for 3 hours. To check clones for the correct insert size, oligonucleotide primers

corresponding to the plasmid's T7 and SP6 transcription domains were used The total PCR reaction volume was 50 μL including 25 pmol of each primer, 0.2 mM of each dNTP, 1x MasterTaq PCR Buffer (Ependorf, Westbury, NY), 1x TaqMaster additive (Ependorf, Westbury, NY), and 0.5 units of MasterTaq Enzyme. The thermocycler program included a denaturing time of five minutes at 95 °C, followed by 30 cycles of one minute at 95 °C, one minute at 50 °C, four minutes at 72 °C, followed by a final extension time of five minutes at 72 °C. PCR products were electrophoresed at 60 V for 45 minutes on a 1.5% high melting temperature agarose gel with a 10,000 bp ladder to examine the quality and quantity of each reaction.

To isolate plasmids, clone cultures that had been successfully screened for the correct insert were incubated in 900 μL of LB-amp at 37 °C and 150 rpm for 48 hours. Following incubation, plasmids were isolated from each clone culture using the QIAprep Spin Miniprep Kit following manufacturer's protocol (Qiagen, Valencia, CA). After isolation, the quality and quantity of each plasmid were examined by subjecting five μL of each sample to electrophoresis on a 1% high melt agarose gel at 80V for 60 minutes. Successful plasmid isolations were sequenced at CFAR using oligonucleotide primers based on the plasmid's SP6 and T7 transcription domains Forward and reverse sequences were assembled using CodonCode Aligner version 4.2.3 (CodonCode Corporation, www.codoncode.com) to generate a consensus sequence for each clone.

16s rRNA Phylogeny

The 16s rRNA gene sequences were aligned using Geneious version 6.1.7 (Biomatters, www. geneious.com) and sequences were edited from approximately 1450 to 1400 base pairs. These sequences were then further aligned with homologous

sequences of other identified organisms found in GenBank Many sequences found in Genbank had had greater than 98% pairwise identity to multiple organisms and these results were preferentially selected for organisms that were defined down to the species level. Fifty-eight sequences were selected and then aligned with the amplified isolated cultures using MAFFT (102) through Geneious version 6.1.7 (Biomatters, www. geneious.com) with scoring matrix = 200PAM/k = 2; gap opening penalty=1.53, and an offset value = 0.123. Resulting alignments were also examined by eye for accuracy.

Phylogenetic Analysis

The program FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel /findmodel.html) was used to determine the best nucleotide substitution model for the sequence alignment. Geneious version 6.1.7 (Biomatters, www. geneious.com) was used to reconstruct 16S phylogenies based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm (103) For Bayesian analyses, the CIPRES Science Gateway (104) was used to run MrBayes version 3.2.2 (105). The model of nucleotide substitution was specified as the general time-reversible model, incorporating a gamma distribution of substitution rates among sites and a proportion of invariant sites (GTR+I+G). Two methanogens were added to the alignment and constrained to form an outgroup. A relaxed clock model was implemented by specify the independent gamma rates relaxed-clock model with a birth-death process. The chain length was set to 10,000,000 generations, with 4 chains implemented in each of 4 simultaneous runs. Each of the 16 total chains was sampled every 500th generation. After 10,000,000 generations, the average standard deviation of split frequencies reached .01; a value less than 0.05

indicated that the chains had converged on an optimal set of phylogenies. All parameters reached a Potential Scale Reduction Factor = 1.00 with a minimum estimated sample size ranging from 159 to 11,995. After a burn-in of 25%, the trees sampled by each of the 16 chains were summarized into a consensus phylogeny. Additionally, RAxML Blackbox (106) was used to reconstruct a16S phylogeny through a maximum likelihood approach; the default settings of this web-accessible program were used. Phylogenetic trees were viewed and edited in FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Statistical Analysis

Correlations between phylogenetic relationships, microenvironments in the BSF, and phenotypes were tested using Arbor (107). Phylogenetic signal exists when more closely related organisms share more similar phenotypes. Phylogenetic signal tests were based on Pagel's lambda (108), using the functions fitDiscrete (for the discrete variables depth, morphology, setting) and fitContinuous (for the continuous variables doubling time, stationary phase, peak optical density); these functions were calculated in Arbor (107) based on R version 2.14.1 (109) package Geiger (109). The phylogeny of Haloarchaea in the BSF was transformed using a lambda value of zero to obtain the likelihood of the null hypothesis of no phylogenetic signal. The negative log likelihoods of the original and transformed phylogenies were compared using a likelihood ratio test approximated by a chi-squared distribution

Analysis of variance (ANOVA) was used to examine the impact of microenvironment (setting) on several continuous variables (doubling time, stationary phase, peak optical density of bacterioruberin) through R version 2.14.1 (109).

Significant ANOVAs were followed by pairwise post-hoc t-tests implemented with a Bonferroni correction.

CHAPTER 3

RESULTS AND DISCUSSION: CHARACTERIZATION OF ARCHAEAL COMMUNITY WITHIN THE BONNEVILLE SALT FLATS

Objectives

This study examined and characterized the previously unknown life that exists in the Bonneville Salt Flats. The objectives of this thesis were 1) to show that life, specifically Halophilic Archaea, exists within in the Bonneville Salt Flats (BSF) 2) to link each isolate to its native niche in the BSF through phylogenetic analysis to examine how environmental pressures affect the diversity of genera in a range of microenvironments and 3) to analyze the effect micro-environmental forces (setting) have on specific phenotypes (growth and pigment presence) of the different isolates.

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Results

Identification of Haloarchaea Based on 16s rRNA genetic sequence

Samples from differing locations within the Bonneville Salt Flats were used to inoculate MGM media. All samples and locations showed growth, with all sampling locations giving rise to many different colony types. The colonies were streaked to isolation and glycerol stocks were made of the pure cultures. Thirty isolates representing all sampling locations were selected for characterization. These were comprised of five random isolates from each of the six microenvironments sampled (smooth surface, crack, upwelling, upwelling deep {UPI}, large upwelling, large upwelling deep). DNA was successfully extracted from all isolates and the respective 16s rRNA genetic sequences of all isolates was successfully amplified and isolated. 16s rRNA genetic sequences for all isolates except for #4 (from smooth surface) and #30 (from UPI) were sequenced. Isolates #14 (upwelling), #19 (large upwelling), #20 (large upwelling), #22 (large

upwelling deep), and #24 (large upwelling deep) were further cleaned using a plasmid vector, and isolate #19 clones showed 16s rRNA genetic sequences belonging to two different genera. The sampling group (excluding #4, #30, and both #19 clones) included 22 isolates belonging to the Genus *Haloarcula*, six isolates belonging to the Genus *Halorubrum* and one isolate belonging to the Genus *Halobacterium* (Figure 1).

Growth Curves and Pigment Presence

All isolates showed an optimal growth temperature of 40°C, with visible growth on solid MGM plates shown within 48-60 hours. The growth curves of all isolates were measured to stationary phase and their doubling times were calculated. Doubling times were calculated using the equation: log(2) / slope of linear portion of growth curve. Each location will be presented separately, followed by a more general discussion.

Surface The isolates found on the surface of the flats varied in time taken to reach stationary phase and doubling time (Figure 2a, Figure 3a). Isolates #2, #3, and #4 all reached their respective stationary phase within 55 hours and had similar doubling times (7.0- 7.5 hours) (Figure 2a, Figure 3a). Isolates #1 and #5 were the outliers in this group, with both taking over 55 hours to reach stationary phase and isolate #5 taking over 70 hrs. Isolates #1 and #5 also had different doubling times (11.0-11.5 hours) from the other three surface isolates. All five isolates showed a spectral signature for bacterioruberin all with a similar optical density, isolate #1 showed a slightly greater density that the rest (Figure 4a).

Crack Isolates from cracks found in the salt crust varied in both the time it took to reach stationary phase and in doubling time (Figure 2b, Figure 3b). Isolates #7, #8, and #9 all reached stationary phase in less than 60 hours. Isolates #6 and #10 took the longest time to reach their stationary phase, both isolates took over 60 hours to reach their stationary phase with isolate #6 taking over 70 hours. Isolates #9 and #10 both had doubling times of approximately nine hours and isolate #8 had a doubling time of 10.6 hours. Isolate #6 and #7 were the outliers in doubling time for the crack isolates, with #6 having a doubling time of 15.4 hours and #7 having a doubling time of 6.7 hours. All five isolates showed spectral signature for bacterioruberin (Figure 4b) with variability in optical density. Isolates #6, #7, and #8 seemed to have a distinctly higher optical density of bacterioruberin than did isolates #9 or #10.

Upwelling Isolates from an upwelling found pushing up to the salt crust varied greatly in both the time it took to reach stationary phase and in doubling time (Figure 2c, Figure 3c). Isolates #15 and #11 reached their respective stationary phases the fastest, with #15 taking approximately 55 hours and #11 taking approximately 60 hours. Isolates #12, #13, and #14 all took approximately 70 hours to reach their individual stationary phases. Isolates #12 and #15 had a doubling time of eight hours and isolates #11 and #13 had similar doubling times of 11.2 hours and 10.7 hours, respectively. Isolate #14 had the greatest doubling time of 17.2 hours. All five isolates showed a spectral signature for bacterioruberin (Figure 4c) with one outlier in optical density. Isolate #13 showed a much lower optical density for bacterioruberin than the rest of the isolates.

Large Upwelling Isolates from a large upwelling found pushing up to the salt crust showed much lower variability in both time to reach stationary phase and doubling time (Figure 2d, Figure 3d). Isolates #16 and #17 took between 50 and 55 hours to reach their individual stationary phase and isolates #18, #19, and #20 took between 62 and 70 hours to reach stationary phase. Isolates #16 and #17 were close in doubling time with #16 taking 6.5 hours and #17 taking 6.9 hours. Isolates #18 and #19 were almost identical, with doubling times of 5.2 and 5.3 hours, respectively. Isolate #20 had the fastest doubling time of the large upwelling group with a doubling time of 4.1 hours. All five isolates showed spectral signature for bacterioruberin (Figure 4d) with variation in optical density. Isolates #18, #19 and #20 all showed a distinctly higher optical density of bacterioruberin than did either isolate #16 or #17.

Large Upwelling Deep Isolates from deeper within the large upwelling found pushing up to the salt crust showed a much lower diversity in both the time it took to reach stationary phase and in doubling time (Figure 2e, Figure 3e). Four isolates #21, #22, #24, and #25, all took 70 hours to reach stationary phase, where as isolate #23 took over 100 hours. Isolates #21, #22, #24, and #25, had doubling times between 8.2 and 11.6 hours where as isolate #23 had a doubling time of 24.3 hours. All five isolates showed spectral signature for bacterioruberin (Figure 4e) with a variation in optical density. Isolate #23 had a much higher optical density than the isolates #21, #22, #24, and #25.

Upwelling 2nd Layer (UPI) Isolates from the upwelling deep showed low variability in both time to reach stationary phase and doubling time (Figure 2f, Figure 3f). All isolates

reached stationary phase between 62 and 72 hours with isolate #30 growing the fastest and isolate #26 growing the slowest. Doubling times for all isolates had a range from 3.4 hours to 4.2 hours with isolate #29 having the fastest doubling time and isolate #29 having the slowest doubling time. Only three of the five isolates showed spectral signature for bacterioruberin (Figure 4f), isolates #29 and #30 showed no bacterioruberin presence. The other three isolates (#26, #27, #28) all showed similar peak optical densities for bacterioruberin.

Phylogenetic Trees

The program Findmodel showed that a General Time Reversible with gamma model (Bayesian) was the best fit for the provided data. The 16S rRNA gene sequences were used to reconstruct three different species- level phylogeny models: 1) Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based model (Figure 5) 2) RAxML based model (Figure 6) 3) Bayesian approach with and without a relaxed molecular clock model (Figure 7). All three reconstructed phylogenies grouped the three genera into monophyletic clades. The single isolate that was identified as belonging to the Genus *Halobacterium* was found to be in an isolated clade, separate from the other two Genera. The five isolates identified as belonging to the genus *Halorubrum* were also grouped into a single clade, separate from the other two Genera. The 21 isolates identified as belonging to *Haloarcula* were separate from the other two Genera and these isolates were further resolved to at least nine distinct clades, revealing an unexpected amount of diversity within this genus.

Statistical Tests for Phylogenetic Signal

To determine if continuous or discrete characteristics correlated with the Bayesian relaxed-clock phylogeny the R functions fitDiscrete and fitContinuous (109) were used to calculate Pagel's lambda (108). Lambda ranges from zero to one, with a value of zero indicating no phylogenetic correlation and a value of one indicating a perfect correlation between a character and a phylogeny. Most characters tested against the phylogeny showed lambda values below 0.001 with the exception of peak optical density of bacterioruberin, which showed a lambda of 0.53, and morphology, which showed a lambda of 0.09. Almost all the variables had P values at or close to one with the exception of optical density, which had a P value of 0.67.

Table 1.

Statistical Tests for Phylogenetic Signal

	Lambda	**Log Likelihood Observed Lambda**	**Log Likelihood Lambda at Fixed Zero**	**Chi Squared**	**P-Value**
Depth	2.16E-13	-23.43	-23.43	2.15E-12	1.00
Setting	3.52E-147	-48.38	-48.38	3.20E-11	1.00
Stationary Phase	5.03E-216	-96.10	-96.10	5.68E-14	1.00
Doubling Time	3.58E-217	-78.90	-78.90	0	1.00
Pigment Presence	5.44E-15	-4.28	-4.28	6.75E-14	1.00
Optical Density	0.53	-18.42	-18.42	0.18	0.67
Morphology	0.09	-11.33	-11.33	0.003	0.96

Since all P values were greater than 0.05, none of the phenotypic characters tested showed a significant phylogenetic signal for the Haloarchaea phylogeny (Figure 8).

Setting Effect on Phenotype

Analysis of variance (ANOVA), followed by a pairwise post-hoc t-tests with a Bonferroni correction, was implemented in R to test the impact of microenvironment (setting) on several phenotypes (hours to stationary phase, doubling time, peak optical density of bacterioruberin). Setting significantly affected doubling time (P = 0.015); pairwise post-hoc t-tests with a Bonferroni correction showed a significant difference in the doubling time between isolates found in the large upwelling deep and the upwelling deep (Figure 9).

Table 2.

Microenvironment (Setting) Impact on Doubling Time: Pairwise post-hoc t-tests implemented with a Bonferroni correction for Doubling Time, only values under 0.05 included for Bonferroni correction

	Degrees of Freedom	**Sum of Squares**	**Mean of Squares**	**F Value**	**Pr (>F)**	**Large Upwelling Deep**
Setting	5	255.1	51.01	3.683	0.015	-
Residuals	21	290.9	13.85	-	-	-
Upwelling Deep	-	-	-	-	-	0.023

Setting significantly affected the hours it took each isolate to reach stationary phase (P = 0.0451); pairwise post-hoc t-tests with a Bonferroni correction showed a significant

difference in hours it took to reach stationary phase of isolates found in the large upwelling deep and the surface (Figure 10).

Table 3.

Microenvironment (Setting) Impact on Time Taken to Reach Stationary Phase: Pairwise post-hoc t-tests implemented with a Bonferroni correction for Doubling Time, only values under 0.05 included for Bonferroni correction

	Degrees of Freedom	**Sum of Squares**	**Mean of Squares**	**F Value**	**Pr (>F)**	**Large Upwelling Deep**
Setting	5	755.2	155.04	2.767	0.0451	-
Residuals	21	1176.8	56.04	-	-	-
Surface	-	-	-	-	-	0..038

Setting significantly affected the peak optical densities of bacterioruberin (P=0.002); pairwise post-hoc t-tests with a Bonferroni correction showed that the significant relationships were between isolates found in the crack, the large upwelling deep, and the upwelling deep (Figure 11).

Table 4.

Microenvironment (Setting) Impact on Peak Optical Densities of Bacterioruberin: Pairwise post-hoc t-tests implemented with a Bonferroni correction, only values under 0.05 included for Bonferroni correction

	Degrees of Freedom	Sum of Squares	Mean of Squares	F Value	Pr (>F)	Crack
Setting	5	3.516	0.7031	5.449	0.00228	-
Residuals	21	2.710	0.1290	-	-	-
Large Upwelling Deep	-	-	-	-	-	0..038
Upwelling Deep	-	-	-	-	-	0.0021

These phenotypes (hours to stationary phase, doubling time, peak optical density of bacterioruberin) were also compared among settings including only the Genus *Haloarcula*; this smaller analysis yielded similar results. The only difference found was in stationary phase, which was no longer significantly different among settings ($P > 0.05$).

Discussion

This study's finding of the presence of Haloarchaea within the Bonneville Salt Flats is novel in that there have been no studies on the life within the Bonneville Salt Flats. The MGM media (23% salt) used in the study was purposefully used to select for extreme halophiles, specifically to find haloarchaea within the BSF. Future studies could use media with differing levels of salt to better test the overall diversity of the BSF. Lowering the salt content would allow for a broader range of organisms possibly including different domains. The presence of the *Halobacteriaceae* family and more specifically the three genera (*Haloarcula*, *Halorubrum*, and *Halobacterium*) matches'

current literature based on life in salterns and salt flats (49, 65). It is also not surprising that the 16s rRNA genetic sequences average 96-99% similarities within the individual genera (63). *Haloarcula* was the dominant genus (Figure 23) within the sample groups, which is surprising but may be due to a small overall sampling population of 30 isolates. However, this dominance of Haloarchaea is supported by *Haloarcula* diversity within this sample size compared to *Halorubrum*. *Haloarcula* have around 10 species that are recognized worldwide where as *Halorubrum* has close to 30 species officially classified (110). Figure 26 shows that *Haloarcula* resolves into nine distinct clades including two isolates (#14 and #25) that form their own clade, not matching any of the species represented in Genbank. Isolate #25 may be due to low confidence; however, the node from which #14 arises has a posterior probability value of 95%. The BLAST of Isolate #14's 16s rRNA genetic sequence identity also showed a lower match at 95% with four different species. Isolate #14 not matching any of the included identified species or Genbank species, perhaps points to a new species of *Haloarcula*. Further research is needed to support this suspicion. *Halorubrum* only resolved to two distinct clades (Figure 26) with all characterized isolates matching a single *Halorubrum* species. This difference in diversity seems to further support that *Haloarcula* is perhaps the dominant genus within the BSF. This of course will need a much larger sample population to confirm. It is not uncommon however for a single haloarchaea genus to dominate an extremely saline environment, where other environmental factors such as UV damage or extreme cold come into effect (62, 63, 74). This diversity could also be explained by the use of only one genetic sequence of 16s rRNA. Some species found within *Haloarcula* are known to have two distinct 16s rRNA genetic sequences and recent studies have shown

that these sequences can differ up to 5% in their identity (111). The variation found in the genome of a single organism is more than that sometimes found between species of Archaea. *Haloarcula* also has a larger genome than does *Halorubrum* as well as and more transposons (91). This could allow species of *Haloarcula* to more readily transfer key adaptations needed in survive in the BSF through horizontal gene transfer. *Haloarcula's* dominance could be due to many different factors such as predation or horizontal gene transfer (due to more transposons) this is a hypothesis that could be tested in a future study. It is important to note here that a single species within the genus *Halobacterium* was found and paired with other known *Halobacterium* species, forming a clade distinct from the other genera. This sample size of one was deemed insufficient for further analysis or comparison against the other two genera and additional consideration would require a much larger sample size.

Growth overall did not seem to be unique for most of the isolates, for instance the doubling times for isolates #1-15 and #20-25 averaged between 9 hours and 13 hours (Figure 3). The model organisms used for Haloarchaea have similar growth curves, *NRC-1* around eight hours and *Halorubrum lacusprofundi* around nine hours (112). However, isolates #26-30 (upwelling deep) and #16-20 (large upwelling) had much faster doubling times of 3.7 and 5.5 hours, respectively (Figure 3). This is faster than both the aforementioned model haloarchaea and even faster than the model haloarchaea *Haloferax volcanii* (4 hours), which is mainly used due to its uniquely fast growth (112). While this currently seems like a unique characteristic, perhaps fast growth is a more common adaptation to hypersaline environments, such as salt flats, than we currently know of, based on the low amount of information available on these environments.

Bacterioruberin is a pigment known to help in UV protection, along with intra and extra- cellular salts (113). Its presence in the isolates was expected however not all of the isolates had it. Isolates #29 and #30 seemed to be completely devoid of the pigment, they were from the deepest settings so perhaps loss of this pigment is associated with depth. This loss could be from the aforementioned use of extracellular salt as a UV protectant. A larger sample size that includes a larger range of depths is needed to fully test this hypothesis.

Our data clearly shows that there was no significant relationship between phylogeny and the micro-environmental pressures that we tested (Figure 19). This can be seen when looking at the clade made up of #12, #21, #26, #26, #29 (Figure 19). This clade has representatives from multiple environments and has variance in almost all traits. It is important to note that this was a preliminary characterization with both a limited sample size and limited scope of characteristics. The most important characteristic that was not measured in any form is that of differing salinity. It is known that the BSF has active upwelling and annual flooding events, which indicates that the BSF has microenvironments of differing salinity. Differing salinity levels, usually caused by the presence or absence of active water sources, appear to be one of the largest environmental pressures that push the divergence of halophilic archaea (43, 53, 62).

There were clear differences in phenotype that seemed to be significant, such as the previously mentioned variance in doubling time. Phylogenetic signal is not the only thing that could be inferred from the data, micro-environmental pressure's effect on phenotype was also analyzed. We tested both depth and setting against growth and pigment presence. Depth had a slightly higher significance but with less variance and so

setting was chosen as the parameter to test. This finding does mean that depth has no effect on phenotype but a wider range of depths and a larger sample size is needed to better test this hypothesis. Setting (smooth surface, crack, upwelling, upwelling deep, large upwelling, large upwelling deep) was tested against the isolates' doubling time, hours taken to reach stationary phase, and the peak optical density of bacterioruberin. All three relationships had significant differences. Settings relationship to stationary phase showed a significant difference between the surface and the large upwelling deep (Figure 10). The large upwelling deep setting took the longest time to reach stationary phase, which could be explained by the depth or perhaps lower amounts of nutrients. The differing microenvironments also had an effect on doubling time, which showed a significant difference between the upwelling deep and the large upwelling deep (Figure 9). The doubling time of the isolates found inside of the upwelling deep setting (the deepest setting) was exceptionally fast, especially in comparison to the doubling time found in the large upwelling deep. The differing microenvironments showed an impact on the bacterioruberin presence in the isolates (Figure 11). The isolates found in the crack had a high bacterioruberin presence with the isolates in the upwelling deep setting having the lowest amount of bacterioruberin including two that seemed to be missing it. This could be a possible explanation for the quick doubling time found in the upwelling deep isolates. The isolates in the upwelling deep (the deepest setting) likely have less UV exposure due to more extracellular salt coverage (a known UV protectant). This lower UV exposure would decrease the efficiency of bacterioruberin, is a costly complex carotenoid (113). Isolates from a deeper setting who no longer made this pigment would seemingly have a faster doubling time which is what is seen in the isolates from the

upwelling deep. Studies have shown that carotenoids (pigments) directly affect the growth of organisms like cyanobacteria but these studies are usually in relation to nutrient limited media and do not focus solely on carotenoids (114, 115). A future study could focus on the relationship between carotenoids presence and growth in Archaea. This could be done by taking the growth curves of both organisms with bacterioruberin and mutants without it, while using the same nutrient rich media mutant strains.

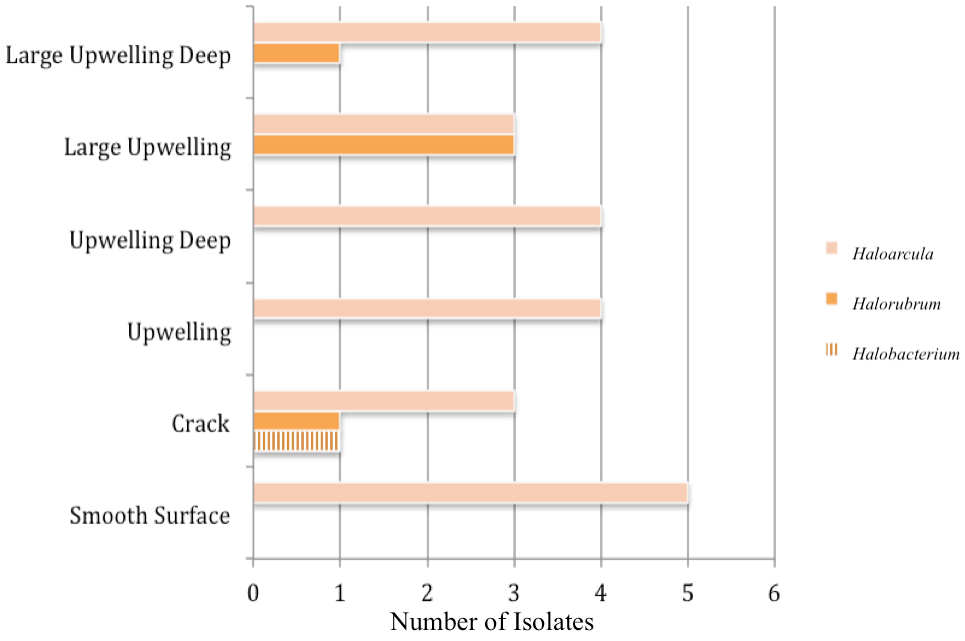

Figure 1. Genera of Isolates Found in Microenvironments

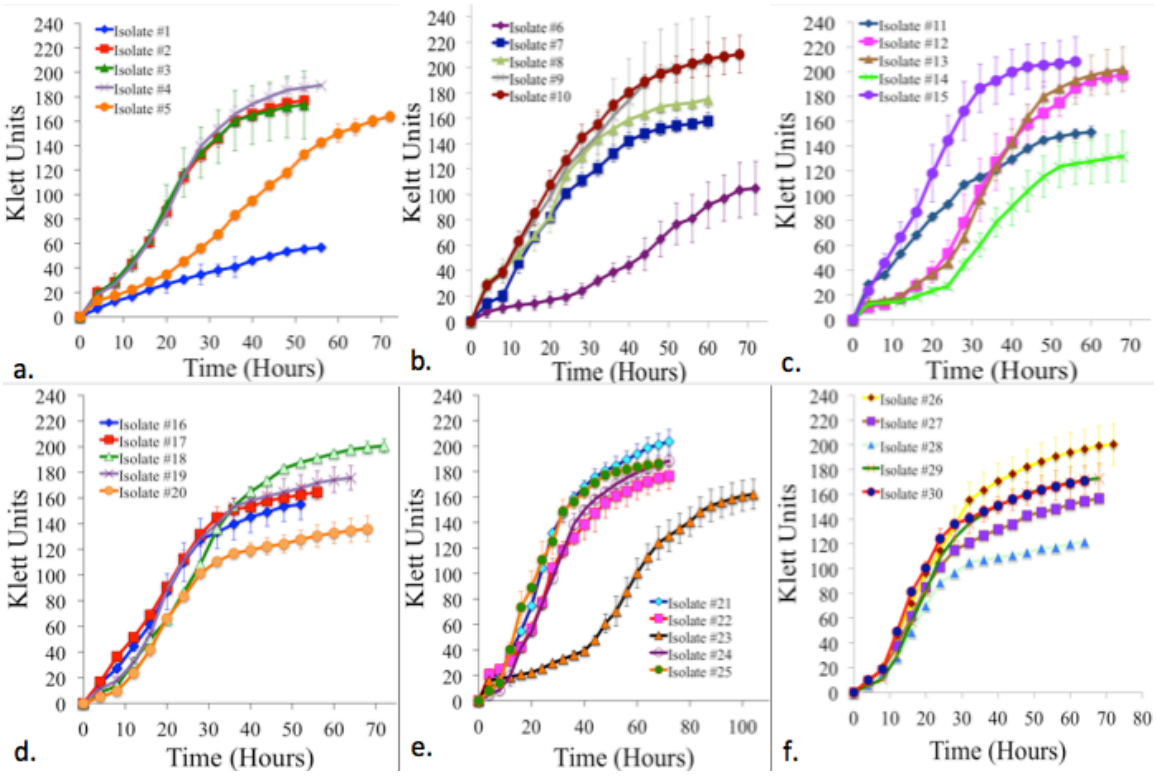

Figure 2. Growth Curves of Isolates: (a.) 1-5 from Surface, (b.) 6-10 from Crack, (c.) 11-15 from Upwelling, (d.) 16-20 from Large Upwelling, (e.) 21-25 from Large Upwelling Deep, (f.) 26-30 from Upwelling Deep.

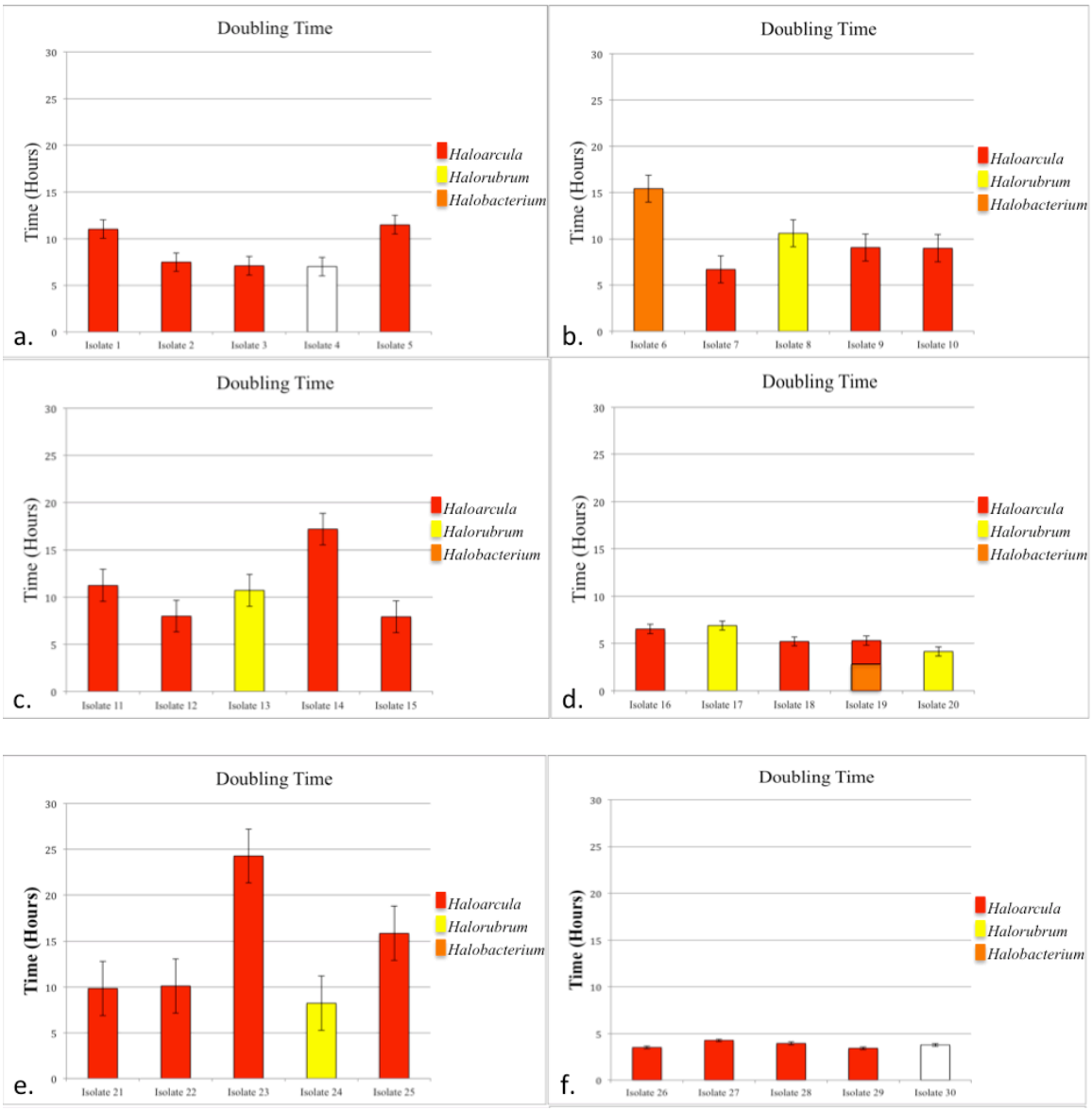

Figure 3. Doubling Time of Isolates: (a.) #1-5 from Surface, (b.) #6-10 from Crack, (c.) #11-15 from Upwelling, (d.) #16-20 from Large Upwelling, (e.) #21-25 from Large Upwelling Deep, (f.) #26-30 from Upwelling Deep. Isolates #4 and #30 were not analyzed due to unclean sequences, #19 was found to have 2 different genera.

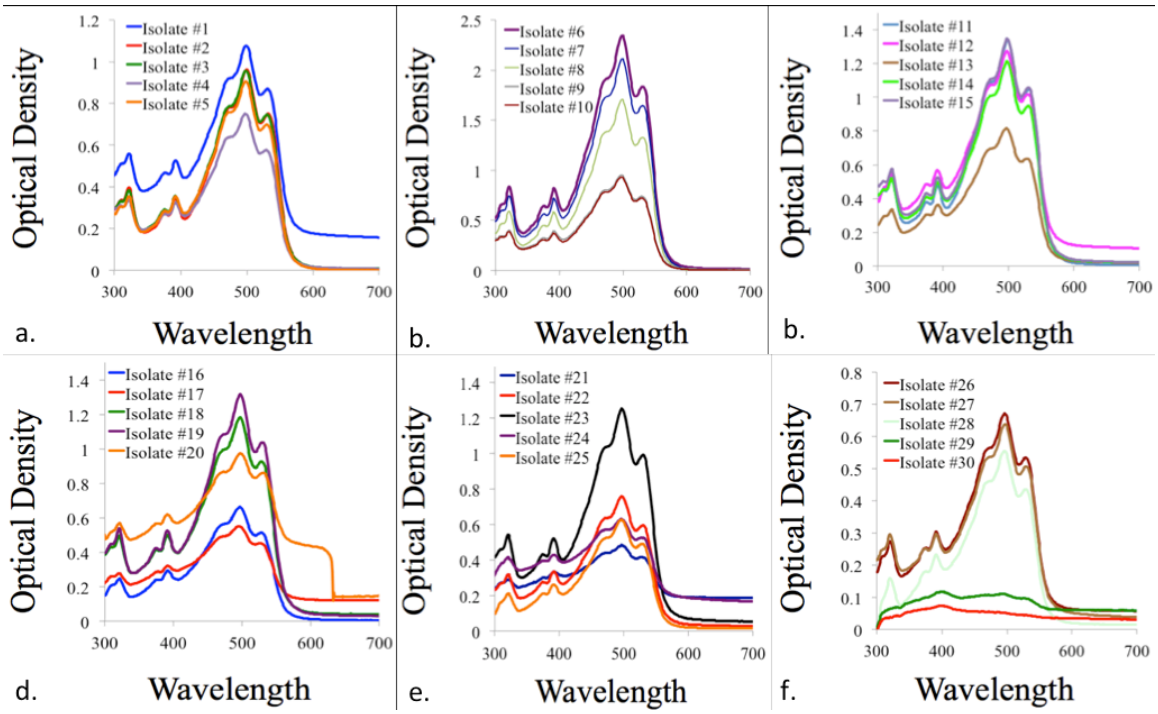

Figure 4. Optical Densities for Bacterioruberin : (a.) #1-5 from Surface, (b.) #6-10 from Crack, (c.) #11-15 from Upwelling, (d.) #16-20 from Large Upwelling, (e.) #21-25 from Large Upwelling Deep, (f.) #26-30 from Upwelling Deep

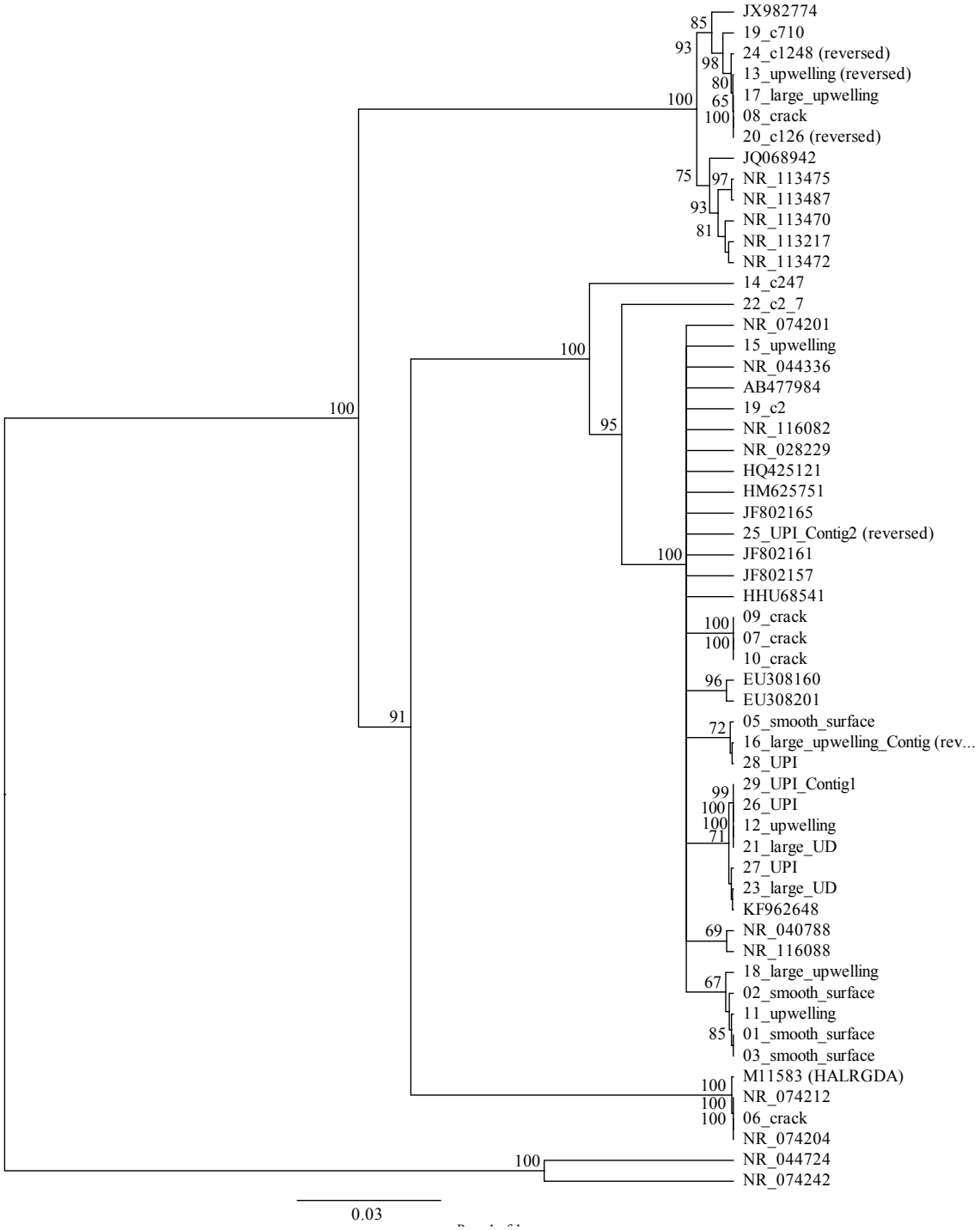

Figure 5. UPGMA Based Tree with bootstrap values less than 60 excluded

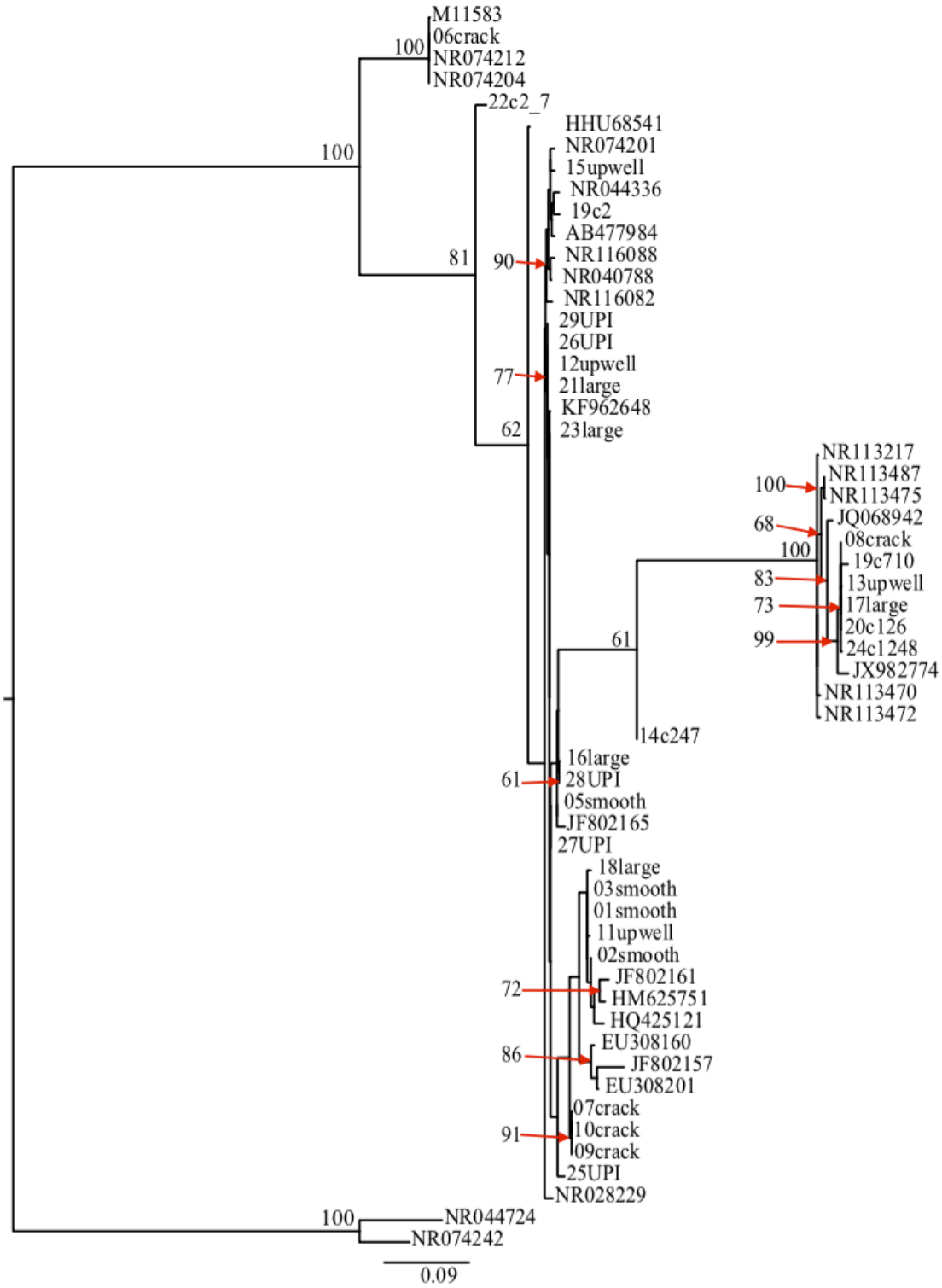

Figure 6. RAxML Based Tree with bootstrap values less than 60 excluded.

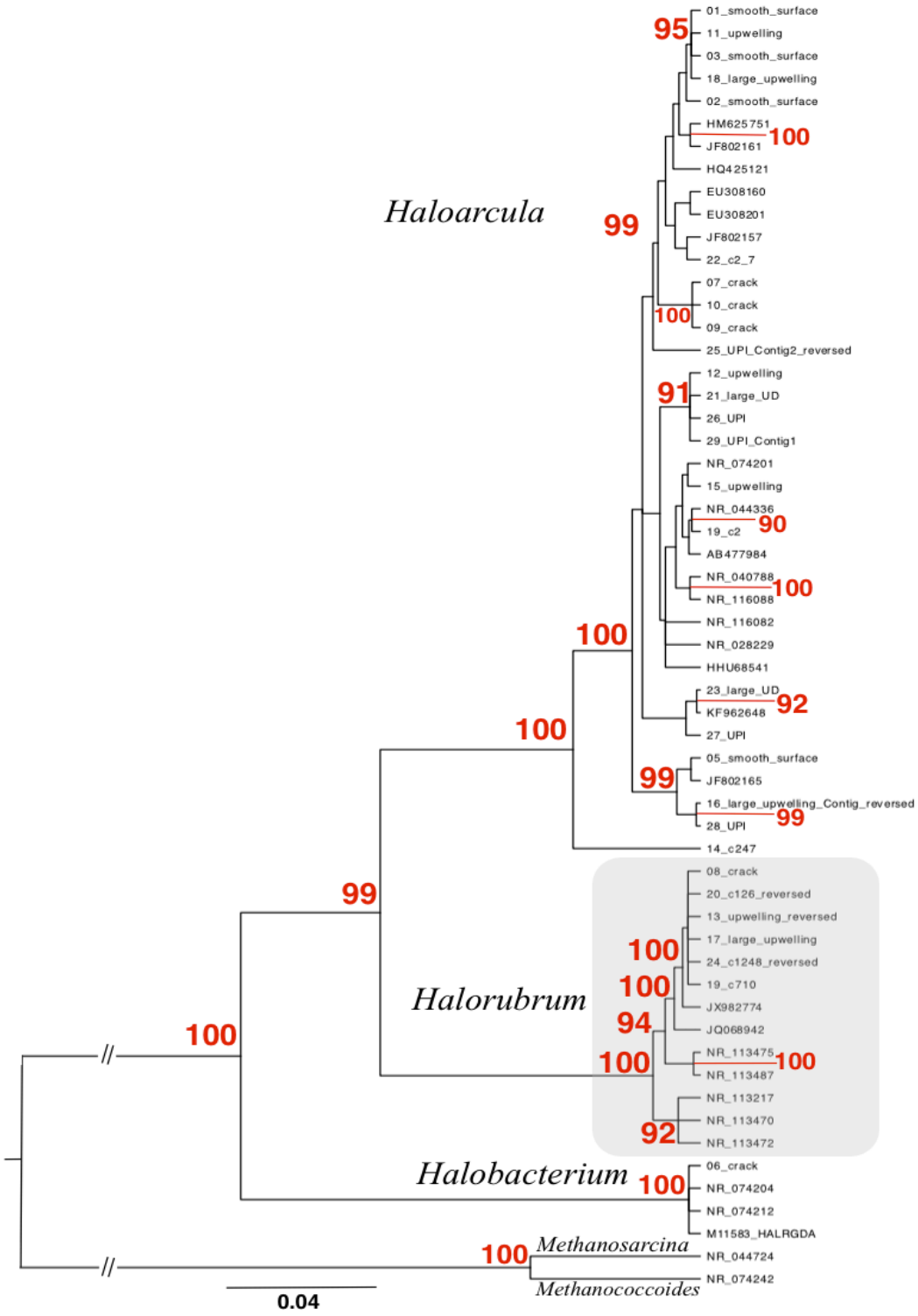

Figure 7. Bayesian Tree with Relaxed Molecular Clock, posterior probability values less than 90 were excluded

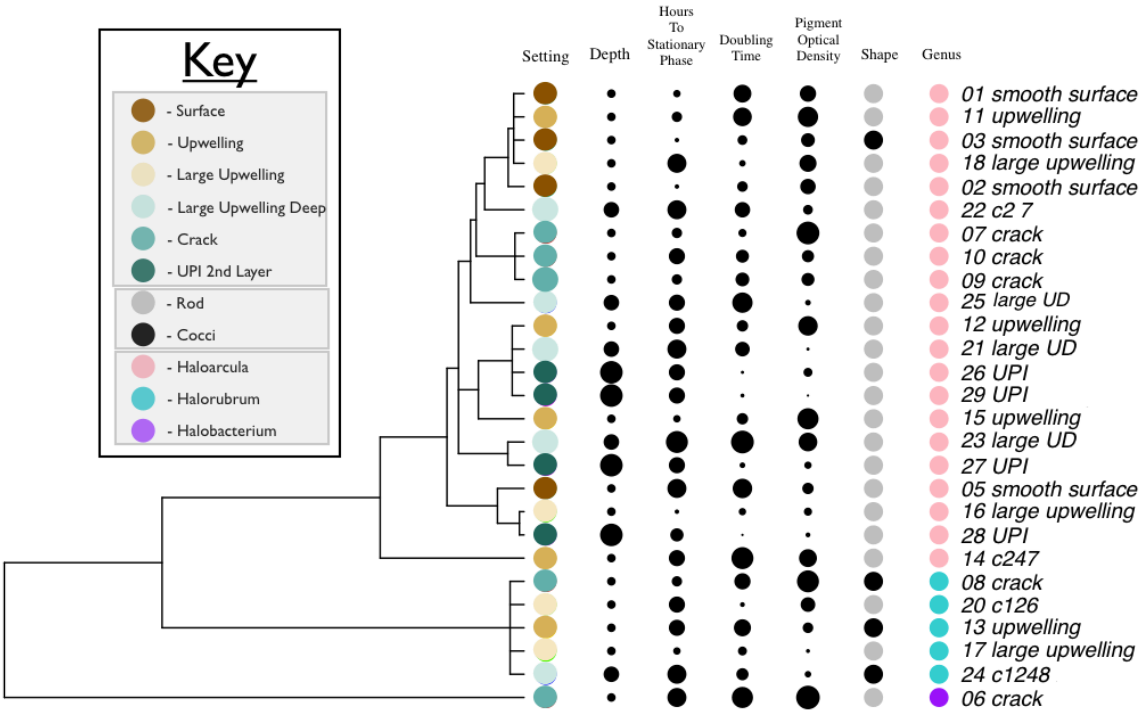

Figure 8. Phylogenetic Tree with Continuous and Discrete Traits

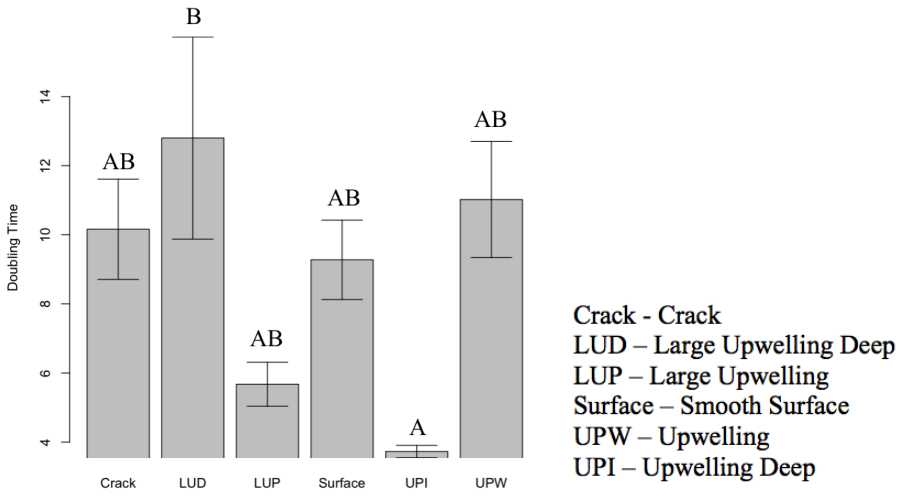

Figure 9. Doubling time in Different Settings

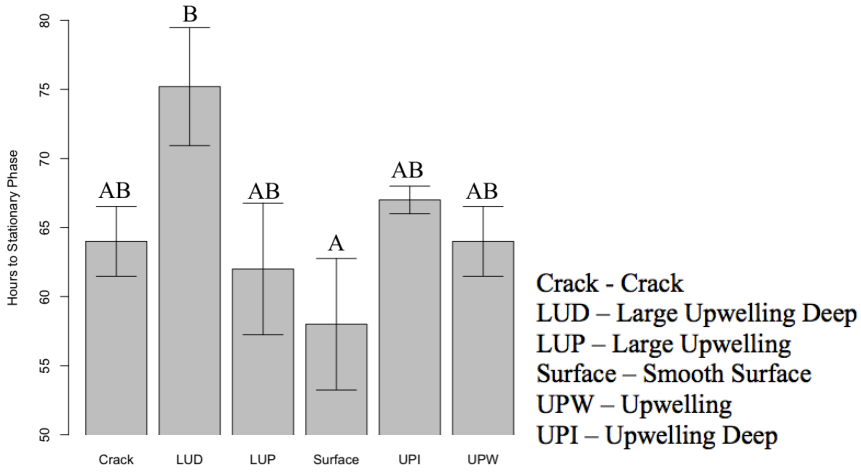

Figure 10. Stationary Phase in Different Settings

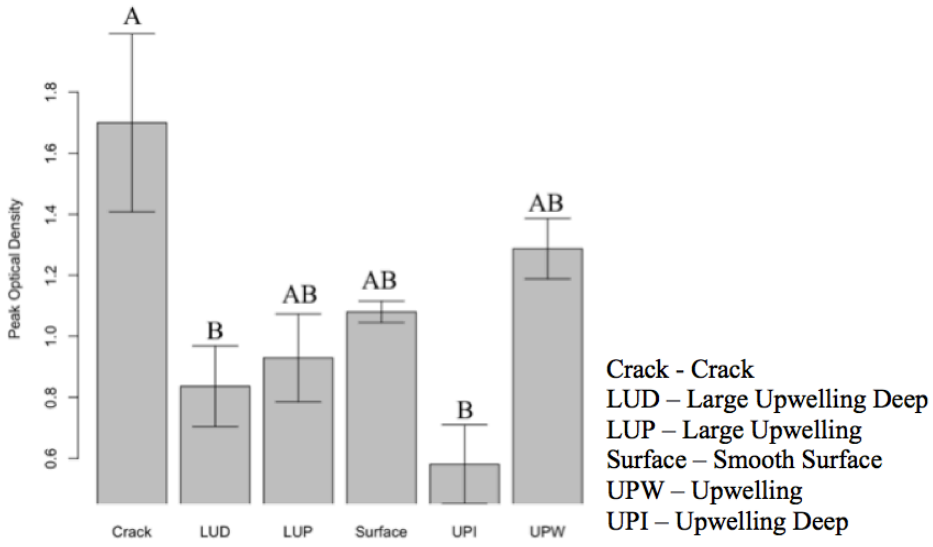

Figure 11. Peak Optical Density of Bacterioruberin in Different Settings

CHAPTER 5

CONCLUSION AND FUTURE WORK

This study discovered the first life to be reported from the hypersaline environment known as the Bonneville Salt Flats. Life found within the BSF was characterized through studying the effect unique pressures within salt flats have on the diversity of life found there. The genera (all within *Halobacteriaceae)* found in the BSF were not unexpected when compared to studies on other hypersaline environments (62) but the variation of diversity between these genera was unpredicted. The genus *Haloarcula* contains about 10 known species, while the genus *Halorubrum* has close to 30 (110). Our preliminary characterization shows that within our sample, *Haloarcula* seems to be the most diverse genus, forming more distinct clades than *Halorubrum* or *Halobacterium*. Further, some isolates (from within the genus *Haloarcula*) formed distinct and unique lineages, which suggests that these isolates may be unique strains, separate from all other known species within the genus. A phylogenetic signal was not found in any of the phenotypic traits tested, with the most significant lambda being 0.53. Several isolates showed unique growth patterns and a distinct absence of a significant pigment used for UV protection. Further analysis demonstrated that there was significant variance in growth and pigment presence between different microenvironments found within the BSF. It seems that depth may represent an important selective factor for halophiles living in the BSF, possibly due to the UV protection of extracellular salt.

This study, being a preliminary characterization, had some limitations that possibly affected outcomes, but could be easily corrected in future studies. First and foremost, a greater sample size of more than 30 isolates, would yield a more accurate representation of the life within the BSF. The genus *Haloarcula* contains multiple distinct 16s rRNA sequences and that these seem to vary in identity by 5% (111). A more accurate future analysis of the organisms found within the BSF will require next-generation sequencing technologies to build phylogenies based on multiple genes. Similar studies are already being done with metagenomics to more accurately separate mixed populations of Bacteria and Archaeal. This method uses the alignment of multiple conserved genetic sequences (20+) such as those from the components of the 30s and 50s ribosome (116). This approach could possibly resolve the *Haloarcula* phylogeny more accurately and resolve the unique clades as either known species or novel strains.

While this study had a limited sampling of characteristics found in the organisms within the unique microenvironments of the BSF, some of these traits were shown to have a significant relationship to their specific environment. The difference in depth between environments seems to encompass a selection pressure that is causing a change in phenotype within the isolates studied. Future studies within the BSF could focus on the affect variance in depth has on the microbial population. This could be done in a more definitive manner than what was done here by using both a larger sample size and a greater variance in depth. This should also be analyzed against phylogeny to test further for a phylogenetic signal. The current literature on halophilic archaea shows that differing salinity levels is another driving force in the their diversity (43, 53, 62). This study lacked the data for differing salinity, which could be included in future studies.

Some isolates within this study showed a distinct absence of the UV protectant pigment bacterioruberin. These isolates where shown to be exclusively in the upwelling deep setting, one of the deepest settings. This limited study showed a possibly significant relationship between depth and the presence of this pigment. Future studies could test the hypothesis that depth within salt flats lowers the need of pigments due to UV protection from a higher level of extracellular salt.

This study was the first to show that microbial life exists within the BSF, specifically members belonging to the *Haloarcula* family *Halobacteriaceae*. Salterns and Salt Flats are some of the least studied environments and the discovery of halophilic life in the BSF is an important step to the initial characterization and understanding of these environments. The understanding of the life found within these hypersaline environments has many important implications for fields such as biotechnology, which continually shows its importance in industry. However, I propose that the most important implication for the finding life in another seemingly desolate place on Earth is in the hope of finding life within Solar System. Finding novel life on another celestial body within our Solar system would fundamentally change our view of the life on Earth. The distances required to reach other planets outside our Solar System are seemingly unachievable. Research on life found within our Solar System is feasible with today's technology, as we have sent spacecraft to every planetary body found within our solar system (98, 117). The question is how to best search for this life? On Earth the presence of liquid water is the single most important factor for finding life (22). Hypersaline liquid bodies seem to provide the most stable aqueous environment in which to find life on another planet, as it has the characteristics of an extremely low freezing temperature and intrinsic UV protection (56,

63). Hypersaline environments have been found within our Solar System including on our closets celestial neighbor Mars (68, 69). The only life we know of is that found on Earth, and there is a large amount of evidence that it emerged from abiotic units (27, 85). If this emergence is a universal principle, as seen in other emergent traits, then one can suppose that life found in hypersaline environments on other planets will be similar to that found on Earth (86). Further understanding of the life within hypersaline environments such as the Bonneville Salt Flats will help us gather the biomarkers we should use to search for life on other planets. The identification of biomarkers for life found in hypersaline environments on Earth is crucial for the needed efficiency of future astrobiological missions. As this study has shown life to exist in the seemingly desolate Bonneville Salt Flats, the search for these biomarkers within the flats can now begin.

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APPENDIX A

METADATA FOR SEQUENCES RETRIEVED FROM GENBANK

NCBI Reference Sequence	*Genus species*	Location of Isolation	Reference
Haloarcula	-	-	-
KF962648	*Haloarcula tradensis*	Fermented fish in Thailand	(118)
NR_074201	*Haloarcula marismortui*	Dead Sea	(119)
NR_044336	*Haloarcula vallismortis*	Salt pools, Death Valley, CA	U - Cui,H.-L., Liu,S.-J.
AB477984	*Haloarcula californiae*	Baja California, Mexico	(120)
NR_116082	*Haloarcula japonica*	Saltern soil at Noto Peninsula, Japan	U - Cui,H.-L., Liu,S.-J.
NR_028229	*Haloarcula amylolytica*	Sediment of Aibi salt lake, Xin-Jiang, China	(121)
JF802161	*Haloarcula amylolytica*	Salt pans Gujarat, India	U - Pal,K.K., Thomas,M.
NR_040788	*Haloarcula quadrata*	Brine pool Egypt, Sinai, sabkha Gavish	(122)
NR_116088	*Haloarcula quadrata*	Brine water Sourthern Sinai, Egypt	U - Cui,H.-L., Liu,S.-J.
JF802165	*Haloarcula argentinensis*	Salt pans Gujarat, India	U - Pal,K.K., Thomas,M.
JF802157	*Haloarcula salaria strain 5A9_DGR*	Salt pans Gujarat, India	U - Pal,K.K., Thomas,M.
HHU68541	*Haloarcula hispanica*	Dead Sea	(123)
HM625751	*Haloarcula sp. IRU1*	Uromia salt lake	U - Asgarani,E., Soudi,M.R.
EU308160	*Haloarcula sp. FC134_15*	Solar saltern Western Greece	U - Tsiamis,G., Katsaveli,K.
EU308201	*Haloarcula sp. FC168_41*	Solar saltern Western Greece	U - Tsiamis,G., Katsaveli,K.
HQ425121	*Archaeon BC29*	Aran-Bidgol Salt Lake, Iran	(124)
Halorubrum	-	-	-
JX982774	*Halorubrum tebenquichense*	Salterns of Sfax, Tunisia	U - Ghanmi,F., Carre-Mlouka,A.
JQ068942	*Halorubrum coriense*	Daishan Solar Saltern, East China	U - Yang,D. and Chen,M.
NR_113217	*Halorubrum ezzemoulense*	Water sample Ezzemoul sabkha, Algeria	U - Minegishi,H., Echigo,A.
NR_113470	*Halorubrum arcis*	Ayakekum salt lake, Western China	U - Minegishi,H., Kamekura,M.
NR_113472	*Halorubrum chaoviator*	Evaporation Pond Baja California, Mexico	U - Minegishi,H., Kamekura,M.
NR_113475	*Halorubrum distributum*	Salt soil crust in Turkmen, USSR	U - Minegishi,H., Kamekura,M.
NR_113487	*Halorubrum terrestre*	Salt crust, Ashkhabad, USSR	U - Minegishi,H., Kamekura,M.
Halobacterium			
NR_074204	*Halobacterium salinarum*	Salty pond in the Arabian desert	(125)
NR_074212	*Halobacterium sp. NRC-1*	N/A	(126)
M11583_(HALRGDA)	*Halobacterium halobium*	N/A	(127)
Outgroup	-	-	-
NR_044724	*Methanosarcina acetivorans*	Marine sedimentint La Jolla, California	(128)
NR_074242	*Methanococcoides burtonii*	Ace Lake water, Antarctica	U - Copeland,A., Lucas,S.

Recognized Genus and Species from NCBI Genbank, unpublished references are identified by a U followed by first and second authors.